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**Assessment of the biocompatibility of PHB and P(HB-HV)**

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ASSESSMENT OF THE BIOCOMPATIBILITY OF PHB AND P(HB-HV).

THESIS

Submitted by Joanne Elizabeth Kennedy, B.Sc., for  
the degree of Doctor of Philosophy  
of the University of Bath.

1990

This research has been carried out in the School of Pharmacy and Pharmacology of the University of Bath under the joint supervision of Dr. C.W. Pouton and Dr. L.J. Notarianni.

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## Contents.

<u>CHAPTER 1. Introduction</u>	<u>Page no.</u>
1.1. Applications of biomedical polymers.	1
1.2. Nonbiodegradable polymers for use in anatomical/ organ replacement.	2
1.3. Nonbiodegradable polymers for use as controlled drug delivery devices.	5
1.4. Biodegradable polymers for use in surgery.	9
1.5. Biodegradable polymers as controlled drug delivery devices.	11
1.6. Poly D-3-hydroxybutyrate (PHB) and copolymers with 3-hydroxyvalerate (P(HB-HV)).	13
1.6.1. Synthesis and structure.	13
1.6.2. Degradation of PHB and P(HB-HV).	16
1.6.3. Drug release from PHB and P(HB-HV) copolymers.	18
1.6.4. Biocompatibility of PHB.	20
1.7. Biocompatibility.	20
1.7.1. Biocompatibility and biofunctionability - Definitions.	20
1.7.2. Criteria for biocompatibility testing.	22
1.7.3. Development of toxicity programmes.	26
1.7.4. Present guidelines for safety and toxicity testing of biomaterials.	27
1.8. Scope of present study.	31
<u>CHAPTER 2. Materials and Methods.</u>	32
2.1. Cell culture.	33

2.1.1. Equipment.	33
2.1.2. Cell line.	35
2.1.3. Cell culture materials.	36
2.1.4. General methods - Gases and gassing procedures.	38
2.1.5. Cell culture methods - Maintenance of cell line.	39
2.1.6. Preparation of cell suspensions from monolayer cultures.	39
2.1.7. Determination of cell suspension density.	40
2.1.8. Subculture routine.	41
2.1.9. Cell storage.	42
2.1.10. Cell recovery from storage.	42
2.1.11. Dilution plating.	42
2.1.12. Staining.	43
2.1.13. Determination of the growth parameters of CHO-K1 cells.	43
2.1.14. Construction of growth curves.	46
2.1.15. Replica plating.	47
2.1.16. Effect of osmolarity and pH.	49
2.2. Assessment of macrophage activation.	50
2.2.1. Materials.	51
2.2.2. Production and collection of macrophages.	51
2.2.3. Macrophage cultures.	52
2.2.4. Coverslip cultures.	53
2.2.5. Macrophage activity assays.	53
2.2.6. Cell viability assay.	53
2.2.7. Scanning electron microscopy.	54
2.3. Plasma protein and platelet adsorption.	55
2.3.1. Materials.	55

2.3.2. Polymer film preparation.	56
2.3.3. Buffer preparation.	56
2.3.4. Protein preparation.	56
2.3.5. Preparation of radiolabeled protein.	57
2.3.6. Separation of labeled protein from unreacted iodide.	57
2.3.7a). Determination of free iodine.	58
2.3.7b). Calculation of the efficiency of iodination.	58
2.3.8. Rinse protocol.	58
2.3.9. Experimental methods. - Preferential adsorption.	59
2.3.10. Adsorption isotherms.	60
2.3.11. Competitive adsorption.	60
2.3.12. Preparation of platelet suspension and isotope labeling.	60
2.3.13. Platelet adhesion experiments.	61
2.4. Assessment of soft tissue inflammatory response: <i>in vivo</i> .	62
2.4.1. Materials.	62
2.4.2. Methods.	63
2.4.2.1. Preparation of injections.	63
2.4.2.2. Histological studies.	63
2.4.2.3. Assessment of increased vascular permeability.	64
2.4.2.4. Assay of cellular activities.	64

### CHAPTER 3. The preliminary assessment of the biocompatibility of PHB, P(HB-HV) and their monomers using cell culture.

3.1. Introduction.	67
3.2. Results.	71
3.2.1. The effect of PHB, P(HB-HV) and their monomers on the	71

growth parameters of CHO-K1 cells.

3.2.2. Replica plating.	85
3.3. Discussion.	87
3.4. Conclusions.	92

#### CHAPTER 4. The effect of PHB, P(HB-HV) and their degradation products on activation of the macrophage.

4.1. Introduction.	93
4.2. Results.	96
4.2.1. Quantification kinetics of macrophage accumulation <i>in vivo</i> .	96
4.2.2. Effect of particulate concentration on macrophage accumulation.	99
4.2.3. Effect of biomaterials on the activation of the macrophage <i>in vitro</i> .	99
4.2.4. Cytotoxicity as shown by the LDH assay.	103
4.2.5. Scanning electron microscopy.	103
4.3. Discussion.	109
4.4. Conclusions.	116

#### CHAPTER 5. *In vitro* plasma protein and platelet interactions with PHB and P(HB-HV) in assessment of their blood compatibility.

5.1. Introduction.	117
5.2. Results -Preferential adsorption.	121
5.3. Adsorption isotherms.	121
5.4. Competitive adsorption.	127

5.5. Platelet adsorption.	135
5.6. Discussion.	138
5.7. Conclusions.	147

CHAPTER 6. *In vivo* biocompatibility studies. Assessment of  
the soft tissue response.

6.1. Introduction.	148
6.2. Results.	152
6.2.1. Macroscopic observations.	152
6.2.2. Histological observations.	152
6.2.3. Effect on vascular permeability.	160
6.2.4. Effect on alkaline and acid phosphatase enzyme activity.	162
6.3. Discussion.	184
6.4. Conclusions.	191

CHAPTER 7. Evaluation *in vitro* and *in vivo* of PHB as a  
biodegradable drug delivery system.

7.1. Introduction.	192
7.2. Materials.	196
7.3. <i>In vitro</i> release experiments.	197
7.3.1. Matrix preparation.	197
7.3.2. Experimental.	197
7.3.3. Inulin assay.	198
7.4 <i>In vivo</i> release experiments.	198
7.4.1. Matrix preparation.	198
7.4.2. Implantation technique.	198
7.4.3. Inulin assay for determination of release kinetics <i>in vivo</i> .	199

7.5. Results.	200
7.5.1. <i>In vitro</i> release kinetics.	200
7.5.2. <i>In vivo</i> release.	211
7.6. Discussion.	216
 <u>CHAPTER 8. Concluding discussion.</u>	 224
 Appendix 1.	 239
References.	240

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## Summary

Biodegradable polymers have important uses both in surgery and as drug delivery devices. Bacterially produced poly D-3-hydroxybutyrate (PHB) and its copolymers with poly 3-hydroxyvalerate (P(HB-HV)) have emerged as new candidates for use in surgery, e.g. bone replacement and skin grafting, and use as controlled drug delivery devices.

Current literature available on the biocompatibility of these polymers and their degradation products is limited. Chapters 3-6 describe a number of *in vitro* and *in vivo* techniques used to assess their biocompatibility in soft tissue.

Chapter 3 describes the effect of PHB, P(HB-HV) and their monomers on the growth parameters of CHO-K1 cells. No significant effect on cell growth when treated with PHB and P(HB-HV) was observed. However at monomer concentrations  $>10\text{mg/ml}$  (HB) and  $>15\text{mg/ml}$  (HV) cell cytotoxicity was observed. This was believed to be due to an osmotic effect.

Chapter 4 describes the effect of the polymers and their monomers on macrophage accumulation number when injected intraperitoneally into CLFP mice. Significant increases ( $p<0.01$ ) in macrophage accumulation number was evident in the PHB and P(HB-HV) injected mice over days 6-8 when compared to injection of vehicle alone. The effect on macrophage activation, as indicated by PGE<sub>2</sub> production and release, was assessed. The polymers and monomers significantly increased ( $p<0.01$ ) PGE<sub>2</sub> production and release during the first 15 minutes of culture when compared to control cultures, but these levels decreased

to control levels within 24 hours. The effect of the polymers and monomers on macrophage cytotoxicity was also assessed using LDH (lactate dehydrogenase) production and release as a marker. Significant increases in LDH production and release was only observed the with the monomers. The effect was again believed to be osmotic.

Blood compatibility of PHB and P(HB-HV) with reference to reported blood compatible materials was assessed using plasma protein and platelet adhesion experiments. Significant increases ( $p < 0.01$ ) in fibrinogen adsorption and platelet adhesion was seen on P(HB-HV) films when compared to PHB and the reference materials.

Chapter 6 investigated the effect of PHB, P(HB-HV) and their monomers on the *in vivo* soft tissue inflammatory response using histological, vascular permeability and enzyme histochemical methods of assessment. Generally an acute inflammatory effect was evident which subsides after 14 days. High concentrations of the monomers were well tolerated.

Finally the *in vitro* and *in vivo* release kinetics of inulin from PHB P(LA-GA) and PLA melt extrusions were investigated. At physiological and accelerated conditions *in vitro* drug release appeared to follow zero-order kinetics. However drug release *in vivo* from the PHB/inulin melt extrusions was negligible.

Hypotheses for the above observations are discussed in each chapter and comparison of *in vitro* and *in vivo* assessment of polymer biocompatibility.

## CHAPTER 1. Introduction.

### 1.1 Applications of biomedical polymers.

A number of review articles on the use of materials in medicine and dentistry have suggested that many structural parts of the human body could be replaced by some type of plastic and/or metal device (Miller, 1978; Pines, 1978). The term 'biomaterial' is often designated to such materials and may be defined as "a material that is used in the treatment of patients, and at some stage, interfaces with the tissue for a significant length of time so that the interaction between the tissue and the material is an important factor in the treatment" (Williams, 1981). In 1986 the European Society for Biomaterials convened a consensus development conference on the theme 'Definitions of Biomaterials'. It has since been agreed by the conference members to define a biomaterial as "a non viable material, used in a medical device, intended to interact with biological systems" (Williams, 1988).

The field of biomedical polymers continues to show steady growth in both basic research and application. Such polymers include totally implanted devices such as prosthetic devices, drug delivery devices, and materials used in the mouth for dental treatment, whether placed on the oral mucosa or located in the enamel, dentine or pulp chamber. According to statistics from the University Hospitals of Case-Western Reserve University, U.S.A., 9-15% of autopsies reveal some type of implant in the patient (Anderson, 1981). This number coupled with the number of people worldwide that have dental fillings, dentures and contact lenses, have made the development and application of polymeric biomaterials an increasingly important field (Falcetta, 1974; Tighe, 1976; Andrade, 1976).

The material requirements for such diverse biomedical applications vary markedly according to the application being considered and it is therefore unlikely that one single polymer would be of use in all situations. The breadth of applications in which typical biomedical polymers are involved are listed in table 1.1. Polymeric materials fall into two broad categories, nonbiodegradable and biodegradable, the applications of which are discussed below.

### 1.2. Non biodegradable polymers for use in anatomical/organ replacement.

Non-biodegradable polymers are used in the development of artificial organs, limb and joint replacements, and artificial skin grafts. Possibly the most spectacular area of application is in the production of an artificial organ. The ultimate aim of artificial organ design is such that the organ is capable of implantation into the body whereupon it is able to replace totally the function of the diseased or disabled organ. However, even with the progress made over the last 20 years within the field of biomedical technology, this has yet to be achieved.

One of the major problems encountered with any artificial organ is that of blood compatibility, since virtually all organs either handle blood directly (heart, blood vessels) or come into contact with blood in a membrane exchange reaction (kidneys, lungs). Numerous polymeric systems have been investigated with regard to their blood compatibility. The criteria required for acceptable blood compatibility is described in detail in chapter 5. Polyethylene terephthalate mesh tubing (Dacron), and polytetrafluoroethylene (Teflon) tubings are at present the most acceptable polymers for use in large blood vessel replacement (Kotte-Marcant *et al*, 1989).

Table 1.1Typical Biomedical Non-degradable Polymer Applications.

Artificial heart  
Artificial kidney  
Artificial limbs  
Artificial liver  
Artificial pancreas  
Artificial penis  
Bone cements  
Bone replacement  
Casts  
Catheters  
Contact lenses  
Controlled release drug vehicles  
Cornea replacement  
Dental fillings  
Dentures  
Drainage tubes  
Drug administration devices  
External ear repairs  
Eye lens replacement  
Heart valves  
Hydrocephalus shunts  
Implantable pumps  
Inner ear repairs  
Joint replacement  
Pacemakers  
Plasma extenders  
Plastic surgery  
Reinforcement mesh  
Replacement blood vessels  
Replacement skin  
Soft tissue replacement  
Surgical adhesives  
Surgical tape  
Sutures  
Testicle replacement  
Visual prosthesis  
Wound dressings

Knitted Dacron has been used for over 25 years as an arterial vascular prosthesis in the replacement of diseased large and medium sized arteries (Sauvage *et al.*, 1978), primarily because its porosity allows tissue ingrowth and promotes graft healing, even though Dacron is recognised to be highly thrombogenic (Kotte-Marchant *et al.*, 1986; Hamlin *et al.*, 1978; and Berger *et al.*, 1972). The thrombogenicity of Dacron is not of major consequence in high flow vessels, such as the aorta, but becomes a major factor in small diameter vessels with low flow. Polyether urethanes are now superceeding all previous polymers for use in cardiovascular systems and, in terms of blood compatibility, are at present reported to be the best materials (Lyman, 1974; and Stupp *et al.*, 1977; Ito *et al.* 1989). However the durability of these polymers in cardiovascular systems is required to exceed the life span of the patient and the polymer must exhibit compatibility with the other body fluids and tissues. Therefore much research is still required on the polyetherurethanes in some areas of biomaterial application.

Progress of research in the use of artificial kidneys and lungs has suffered great setbacks in finding a biomaterial which has adequate blood compatibility coupled with good membrane characteristics. The situation with artificial lungs is particularly poor, as blood-membrane interface area is much greater and blood compatibility therefore becomes even more critical (Chien, 1980).

The use of polymers where long term contact with blood does not occur has advanced to a greater extent than those uses which imply long-term contact with blood. A major use of polymers in surgery has been replacement of soft-tissue such as the prosthetic breast and testicles. The major requirements of such soft-tissue replacement polymers include; no adverse reactions with body tissues, no fibrous

in-growth and a consistency similar to the natural material being replaced. To date the major polymer used in such applications has been polydimethylsiloxane (Chien, 1980). Artificial limb and joint replacement, where blood compatibility is not as important as durability and long term stability, has also progressed steadily and is the subject of many review articles of polymers in surgery (Geblein, 1981; Hulbert *et al*, 1973., Charnley, 1976). Plastic can now be used as one of the articular surfaces in an artificial hip or knee, for example in metal/ plastic total joint replacements (total arthroplasties), or as an alternative to metal in joints where only one of the natural surfaces is replaced by an artificial material (hemiarthroplastie). However in both these circumstances there is the possibility that wear particles from the articular surface will accumulate in the synovial membrane or the joint capsule leading to inflammation. (Charnley, 1970). A further use of such polymers is as a cement to interlock a metal prosthesis within a bone. Polymers used as cements include polymethylmethacrylate. However, even this polymer of known biocompatibility (Langer *et al.*, 1981) has been reported to induce tissue necrosis at sites adjacent to the cement (Willert *et al.*, 1974; Feith, 1975).

### 1.3. Nonbiodegradable polymers for use as controlled drug delivery devices.

Recently, a great deal of interest has centered on the idea of utilizing biocompatible polymers to regulate the delivery of pharmaceuticals and veterinary drugs. Controlled release systems have been defined as those which result in the delivery of an active agent from a device to a target site at a rate and for a duration that are controlled by the device itself (Mills and Davis, 1987). Such devices

may offer several important advantages over conventional therapy, these include: 1) maintenance of optimal drug concentrations in body tissues, resulting in greater efficiency of treatment. 2) avoidance of peaks and troughs in drug concentrations resulting in fewer side effects and enhanced safety. 3) ability to administer drugs with narrow therapeutic indices or short biological half lives. 4) greater patient compliance and convenience resulting from the use of fewer administrations per unit time.

A controlled release delivery system is a combination of a biologically active agent with the excipient, a polymeric material. In monolithic devices the drug is uniformly mixed with a polymeric matrix and is present either in the dissolved or dispersed form. For a dissolved drug Fick's law of diffusion applies for the release kinetics (Boer *et al.*, 1987, Langer and Peppas, 1981), whereas the release of dispersed drug can be described by Higuchi's equation, (Higuchi, 1961), (see chapter 7). In the mid 1960's investigations of approaches for incorporating drugs into solid polymers began. In initial studies drugs were placed in the inside lumen of dialysis (Waitz, 1963) or silicone (Folkman and Long, 1964) tubing, or evenly distributed through a polyethylene matrix (Desai *et al.*, 1965). In the 1970's, systems were developed to continuously release large molecular weight drugs ( $M_w > 600$ ) from solid polymers. Ethylene-vinyl acetate and ethylene-glycol methacrylate copolymers and various hydrogels were the most successful in this regard (Langer and Folkman, 1976). A representative of this type of drug delivery device is the Syncro-Mate-B implant, engineered to release norgestamet in the sub-cutaneous tissue at a rate of  $504 \text{ mcg/cm}^2/\text{day}$  for up to 16 days, for oestrus control and synchronization in livestock (Chien, 1980). This implant system uses an ethyleneglycol methacrylate polymer as the



controlling vehicle. A Nitro-Dur system, also of the matrix type is designed to be applied onto intact skin to provide a continuous transdermal infusion of  $500\text{ug}/\text{cm}^2/\text{day}$  of nitroglycerin, in the treatment of angina pectoris. This matrix system uses an aqueous solution of a water-soluble polymer, glycol and polyvinyl alcohol to form a polymer gel and thereby control the release of the active drug.

Other polymeric drug delivery systems include reservoir systems which enclose the drug within an inert polymer membrane (see table 1.2). Although in practice reservoir systems will not deliver drugs at a constant rate either initially, or towards the end of their working life. An initial burst effect is seen due to membrane saturation with the drug following storage (Hadgraft and Guy, 1987), and ultimately a decrease in drug release occurs at the end of the systems life, as the driving force for diffusion through the membrane decreases and the release rate decreases with time (Smith and Lonsdale, 1985). Examples of commercially manufactured reservoir systems include the Ocusert device, which delivers pilocarpine to the eye for the treatment of glaucoma (Stewart and Novak, 1978), and the Progestasert system which was a uterine contraceptive system capable of delivering progesterone to the uterus for the duration of one year (Brenner *et al.*, 1975). Other reservoir systems currently used and with increasing popularity include the Transiderm-Nitro system engineered to achieve a transdermal absorption of  $500\text{ug}/\text{cm}^2/\text{day}$  of nitroglycerin for the relief of angina attacks, and Estraderm which administers a controlled dose of estradiol transdermally over 3-4 days for postmenopausal women. In these reservoir membrane systems the polymeric membrane used to control drug delivery is an ethylene vinyl acetate copolymer (Chien, 1980).

Other nonbiodegradable polymer systems employed as controlled

Table 1.2. Examples of controlled drug delivery systems using polymers.

Disease or treatment	Drug	Polymer	Release rate	Duration	Implant site	Status
Glaucoma	Pilocarpine	Ethylene-vinyl acetate (Ocuser)	20 ug/h 40 ug/h	1 week	Conjunctiva	Currently marketed commercially
Birth control	Progesterone	Ethylene-vinyl acetate (Progestasert)	65 ug/day	1 year	Uterus	Now withdrawn
Motion sickness	Scopolamine	Microporous membrane	0.4 ug/h	3 days	Skin	Currently marketed commercially
Narcotic antagonists	Naltrexone	Lactic glycolytic acid copolymer Polyorthoesters Polyaminoacids	3 ug/h	50 days	Subcutaneous	Has undergone trials in rats
Diabetes	Insulin	Ethylene-vinyl acetate	100 ug/day	1 month	Subcutaneous	Has undergone trials in rats
Immunisation	Antigens	Ethylene-vinyl acetate	0.5 ug/day	6 months	Subcutaneous	Has undergone trials in mice
Dental caries	Fluoride	Poly-2-hydroxyethyl methacrylate		6 months	Back teeth	In Clinical trials
Cancer (Prostate)	Testosterone	Silicone		1 Year	Subcutaneous	In Clinical trials

drug delivery systems include particulate systems which can be injected locally, e.g. into joints for the treatment of arthritis, (Ratcliffe *et al.*, 1984). Recently such systems have shown potential in passive targetting of drugs to organs (Tomlinson *et al.*, 1984). These particulates are varied in design and manufacture and may release their contents as a reservoir, as in the case of microencapsulated systems or more commonly as matrices.

All these implanted nonbiodegradable polymeric drug delivery systems require surgical removal once all the drug has been released, in order to eliminate the potential health risks associated with non-degrading foreign substances remaining in the body for an indefinite period of time. This is neither acceptable or convenient to patients. A new generation of biodegradable polymers has now emerged for use in controlled drug delivery devices and other procedures which themselves degrade with time avoiding the necessity for surgical removal.

#### 1.4. Biodegradable polymers for use in surgery.

The terms bioabsorbable, bioerodible and biodegradable are frequently used interchangeably. The definitions that different authors give to these terms also vary. Williams (1982) defined biodegradation as the biological breakdown of a polymeric material, whereas Gilding (1981) defined the term biodegradable as any polymer that undergoes *in vivo* degradation. Biodegradable polymers have two major applications in surgery: the temporary scaffold and the temporary barrier.

The temporary scaffold includes the absorbable suture. Suture design requires the biodegradable polymer to hold both sides of a wound in close apposition until sufficient collagen synthesis has

taken place to hold the wound together unassisted. Since 70-80% of total collagen synthesis usually occurs within the first three weeks after suturing, the final 20-30% requiring periods of 3-6 months, absorbable sutures therefore required to be structurally stable for at least the first three weeks of implantation. Amongst the most widely used absorbable sutures today are those made from polyglycolate (PGA), marketed as Dexon and a copolymer of lactate and glycolate P(GA/LA), marketed as Vicryl. Within the body these polymers are reported to begin filament breakdown between 21-28 days and are totally eliminated from the body after 80 days. (Reed, 1978; Wilson *et al.*, 1981). The original Dexon sutures were stiff due to the rigidity caused by 6 denier filament of this highly crystalline material. However recently, a 2 denier filament, Dexon-S, was made and had mechanical properties very similar to silk. These biodegradable polymers are biocompatible within the soft tissue that they are implanted and have a mechanical strength superior to that of catgut. (Frazza and Schmitt, 1971; Gilding and Reed, 1981).

Poly-D-L-lactate, (PLA), is amorphous and since it has no dimensional stability is of no use for sutures (Hodge, 1971). It is a hydrophobic polymer and remains *in vivo* for at least 2-3 years when fabricated as plates and screws, (Getter *et al.*, 1972; Leray, 1979). Blood vessels, ureter, bladder, bile duct and heart valve design could, if tailored and fabricated suitably using biodegradable polymers, allow tissue ingrowth and partial regeneration of the natural structure. In the latter cases the polymer is acting initially as a temporary scaffold (Lauritzen *et al.*, 1983).

Biodegradable polymers have also been used as temporary barriers in the fields of tendon, spinal and open-heart surgery. A temporary barrier prevents surgical adhesions, caused by blood

clotting and later fibrosis, occurring between the sliding surfaces of the tendon, or cardiac wall and the pericardial sac. These polymeric barriers remain *in situ* until all the fibrin has been phagocytosed and are then degraded to an innocuous product which is excreted. Such devices have proved to be invaluable aids to the surgeon.

Other potential uses for biodegradable polymers in surgery include materials for absorption of blood during surgery. The use of biodegradable polymers has the advantage that, if the material was inadvertently left in the body, the surgeon would not have to reoperate to retrieve it, since the material would eventually degrade.

Newer applications for biodegradable polymers are those of controlled drug delivery systems, some of which are discussed below and in further detail in chapter 7.

#### 1.5. Biodegradable polymers as controlled drug delivery devices.

In the application of drug delivery, biodegradable polymers when appropriately designed have the advantage that the drug vehicle degrades to a non-toxic product which negates the need for surgical removal once all the drug has been released. Early work on biodegradable polymers concentrated on the release of low molecular weight drugs. Biodegradable implants for the controlled delivery of narcotic antagonists were first described by Yolles *et al.*, (1973). The delivery devices were either drug loaded films or drug loaded polymer powders suspended in a dispersing agent (Yolles *et al.*, 1974, 1975 and Leafe *et al.*, 1974). In a continuation of this work the application of the biodegradable polyester, poly-L-lactate, was extended to a number of different drugs (Yolles *et al.*, 1975). Poly-L-lactate and racemic polylactate were later used for controlled delivery of contraceptives in the form of monolithic devices

(Jackanicz *et al.*, 1973) and as reservoir devices for the delivery of narcotic drugs (Thies, 1976; Mason *et al.*, 1978).

Research is now directed towards the release of macromolecules from these biodegradable polymers. Wise *et al.*, (1987) highlighted the delivery of biological mediators, normally proteins and peptides, using a biodegradable polymer as the vehicle. Polypeptides cannot be given orally as they are deactivated by proteolytic enzymes in the gastrointestinal tract and are therefore administered parentally. However such drugs often have very short biological half lives and frequent injections are required to produce an effective therapy. Biodegradable polymers may therefore be useful in controlling the delivery of these polypeptides and at the same time protect the peptide from degradative effects. Current examples that exist for the controlled drug delivery of peptides and proteins largely utilise the polyglycolate-poly lactate (P(GA-LA) systems. Such a commercial product which was developed using P(GA-LA) copolymers is Zoladex, manufactured by ICI plc for the controlled delivery of an analogue of luteinising hormone releasing hormone (LHRH) (Hutchinson and Furr, 1985). The controlled release of peptides from P(GA-LA) systems has been studied extensively (Sanders *et al.*, 1984, 1985 and 1986; Heller *et al.*, 1987; Kaetsu *et al.*, 1987).

The biodegradable polymer poly D-3-hydroxybutyrate (PHB) and related copolymers poly 3-hydroxybutyrate-valerate (P(HB-HV)) have recently been studied as potential biodegradable drug delivery systems and as a temporary scaffold in surgery. A more detailed description these polymers is given below.

#### 1.6. Poly-D-3-hydroxybutyrate (PHB) and copolymers with 3-hydroxyvalerate (P(HB-HV)).

##### 1.6.1.Synthesis and structure.

- see 1.6.1.2

## 1.6. Poly-D-3-hydroxybutyrate (PHB) and copolymers with 3-hydroxyvalerate (P(HB-HV)).

### 1.6.1. Synthesis and structure.

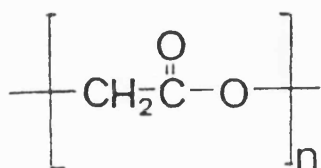
PHB is a naturally occurring polymer, synthesised and metabolised by a range of micro-organisms, including *Azobacter beijerinckii*, *Bacillus cereus* and *Alcaligenes eutrophus*. Its microbial synthesis and metabolism have been studied by many workers (Williamson and Wilkinson, 1958; McRae and Wilkinson, 1958; Alper *et al.*, 1963; Senior *et al.*, 1972 and Emenawa, 1981). PHB is believed to act as an energy reservoir in these bacteria, and is produced in response to an imbalance of growth brought about by nutrient limitation (Dawes and Ribbons, 1964; Dawes and Senior, 1974)

PHB was first isolated and characterised in 1925, by Lemoigne at the Pasteur Institute in Paris and since then it has been extensively studied. It is a highly crystalline thermoplastic with a melting point of approximately 180°C and is structurally related to the aliphatic polyesters poly-D-lactate and polyglycolate (figure 1.1.) As shown, its carbonyl oxygen and methyl group alternate on every other carbon atom along the backbone of the chain. The  $\alpha$  carbon is assymetric and of the  $\beta$  configuration. This structure has been supported by the following chemical evidence :

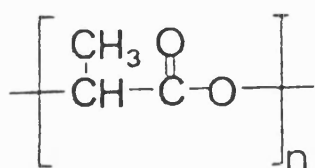
- 1) hydrolysis, saponification or heating of PHB yields 3-hydroxybutyric acid
- 2) the empirical formula corresponds to  $(C_4H_6O_2)_n$
- 3) autolysis yields 3-hydroxybutyric acid (Baptist *et al.*, 1964).

Recent work has shown that some bacteria produce a copolyester of hydroxyvalerate and hydroxybutyrate i.e. P(HB-HV) (Collins, 1982; Holmes *et al.*, 1981). P(HB-HV) can be biosynthesised by culturing the micro-organism in a medium containing a mixture of

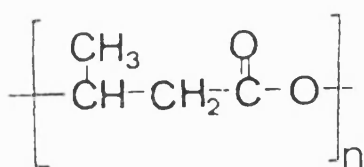
Figure 1.1 :- Chemical structure of related polyesters.



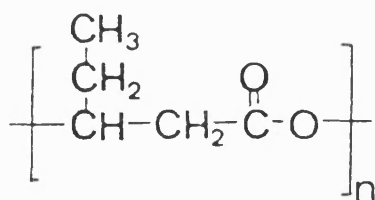
poly (glycolate)



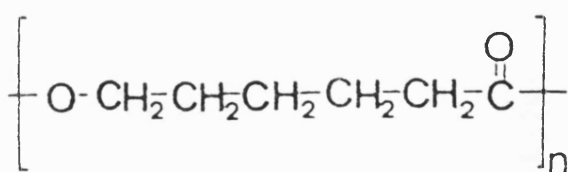
poly (lactate)



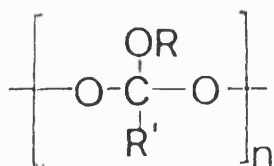
poly (  $\beta$  - hydroxybutyrate )



poly (  $\beta$  - hydroxyvalerate )



poly (  $\epsilon$  - caprolactone )



poly ( orthoesters )



methylacetate. (Holmes *et al.*, 1981). The effect of incorporating HV units into the PHB chain is to modify the crystalline morphology and lower the melting point resulting in a decrease in stiffness but increase toughness (Akhtar *et al.*, 1987).

Literature has shown that PHB and its copolymers ultimately biodegrade to CO<sub>2</sub> and energy in micro-organisms. However in cases of non-enzymatic degradation the degradation product of the homopolymer is 3-D-hydroxybutyric acid, (HB). This is a natural constituent of blood being one of three ketone bodies and its typical concentration in whole human blood is 0.5mg/100ml (Documenta Geigy, 1962). This concentration can greatly increase in states of fasting, ketosis, diabetes and pregnancy, and has been reported to increase to 30g/100ml in severe cases of diabetes, (Shaffer and Marriot, 1913). Such high concentrations of HB in blood appear to be well tolerated and therefore it is unlikely that degradation of the polymer *in vivo* to this product will result in systemic toxicity. The additional degradation product of the copolymers P(HB-HV), 3-D-hydroxyvaleric acid (HV), is not a natural constituent of blood, but because of its structural similarity to HB it is thought to be well tolerated. The rate of biodegradation of PHB and P(HB-HV) depends on the environment in which the polymer is placed; the greatest rate of biodegradation has been reported in anaerobic sewage and well watered soil, slower rates result following subcutaneous or intramuscular implantation in animals, and the rate of degradation *in vitro* is slower still (Holland *et al.*, 1987)

Patents relating to the extraction and use of PHB were filed in the U.S.A. 25 years ago (Baptist, 1962, 1965). Recently PHB has attracted industrial attention as a possible candidate for large scale biotechnological production (King, 1982; Howells, 1982) due to

its high tensile strength, comparable to that of isotactic polypropylene, and its complete biodegradation. PHB has been proposed for use as sutures, gauzes, bone plates, splints, and microcapsules for controlled drug release. Many investigators are now looking at this biomaterial to deliver drugs in a controlled manner similar to biodegradable polyesters such as copolymers of poly(lactate-co-glycolate) marketed as Zoladex, to control the release of LHRH, and similar potent peptides (Hutchinson and Furr, 1987).

#### 1.6.2.Degradation of PHB and P(HB-HV)

The rate of degradation of PHB and its copolymer both *in vivo* and *in vitro* has been investigated by a number of workers. Miller and Williams (1987) studied the degradation of monofilaments of PHB and P(HB-HV) *in vivo* and *in vitro*. Little change in tensile properties was evident in either case although irradiation was found to reduce molecular weight of the polymer and increase its susceptibility to degradation. Increasing the valerate content also increased the rate of degradation by decreasing the sample crystallinity and the resistance to hydrolytic attack correlated with the differences in crystallinity of each of the preparations. Bissery *et al.*, (1984) found no evidence of PHB degradation in their work on the controlled release of a drug from 'biodegradable' PHB microspheres. Holland *et al.*, (1987) have suggested that one possible reason for the relative stability of the drawn PHB monofilaments reported by Miller and Williams (1987) may be due to a high degree of crystallinity, a typical consequence of the extrusion process used to produce monofilament fibres. Holland also showed that the rate of polymer degradation was dependent on the morphological form of PHB observing

differences between solvent cast films, melt pressed discs, injection moulded samples and compressed tablets. These various forms of PHB had different resistances to hydrolytic attack, the least resistant being compressed tablets and then solvent cast films < melt pressed discs < injection moulded samples. The reason being a greater degree in porosity of compressed tablets etc. thereby increasing the effective surface area for degradation. The rate of degradation of PHB is also influenced by pH. An increase in pH increased the rate of degradation suggesting that the degradation mechanism was base catalysed as would be expected for ester hydrolysis (Holland *et al.*, 1987) These authors further showed that the molecular weight of the polymer had a profound influence on the apparent weight loss per unit time. Low molecular weight polymers broke into smaller fragments earlier resulting in more rapid apparent loss of weight. Majid (1987), investigated the hydrolysis of PHB and copolymers P(HB-HV) using solvent-cast films at different values of pH at 37°C. Weight loss was measured and appeared to follow a zero-order pattern. A surface erosion degradative mechanism was then suggested. The rate of degradation increased as the pH was increased and was therefore base-catalysed and not acid-catalysed. All copolymers degraded more rapidly than PHB but the maximum degradation occurred at 12.3mole% PHV. Yasin *et al.*, (1989) blended P(HB-HV) copolymers with polysaccharides to accelerate the rate of hydrolytic degradation. The polysaccharides increased the internal porosity and therefore collapse of the matrix was more rapid. These authors believed that the potential value of these blends extend from surgical devices, such as surgical clips, to drug delivery systems. *In vivo* work by Baptist (1965), showed that PHB films, after 8 weeks implantation into rabbit leg muscle, had begun to break up with an assumed weight

loss, however Frazza and Schmitt (1971) found that even after 90 days implantation *in vivo* no gross changes were apparent.

It can be summarised that degradation of PHB is dependent on both the material characteristics as well as environmental conditions. More carefully controlled experiments are required to describe fully the characteristics of degradation of PHB and P(HB-HV).

#### 1.6.3. Drug release from PHB and P(HB-HV) copolymers.

Korstako *et al.* (1984), prepared PHB matrix tablets as sustained release formulations using 7-hydroxyethyltheophylline (HET) as a model drug. Drug loadings, between 5% and 80% w/w were used and *in vitro* release studies performed in non-sterile 0.9% NaCl at 37°C. Where the drug loading was below 30%, drug release took place over a period of 50 days, but at drug loadings between 60-80% w/w the release was completed within 24 hours. Release of drug occurred initially from the surface then proceeded by diffusion through the aqueous pores formed by water penetration of the matrix of the tablet. The degradation of PHB had no significant effect on the release of HET *in vitro*. When these polymer matrices were implanted subcutaneously into the neck crease of mice the release rate of HET was found to be 2-3 times slower when compared to the *in vitro* release rates.

Bissery *et al.*, (1984) studied the release of an anticancer drug, lomustine 1-(2 chloro-ethyl)-3-cyclohexyl-nitrosourea, (CCNU), from solvent evaporated PHB microspheres, both *in vitro* and *in vivo*. *In vitro*, in buffer of pH 7.2 at 37°C, more than 90% of loaded drug (7.4% w/w) was released in 10 hours. *In vivo* the loaded microspheres were injected intravenously into mice bearing AKR leukemia but had little effect on increasing the life span of the animals.

Juni *et al.* (1986), also prepared PHB microspheres containing the anticancer drug, aclarubicin hydrochloride, using a solvent evaporation method. The release rate of the drug from the microspheres was measured *in vitro*. 10% of the drug was released in 5 days in an isotonic NaCl solution at 37°C. . In an attempt to modify the release pattern of the drug, ethyl and butyl esters of fatty acids with more than 12 and 10 carbon atoms in the acyl chains were included and shown to enhance the release rate of the drug.

Brophy and Deasey (1986) prepared PHB and P(HB-HV) microspheres containing suspended sulphamethiazole using a solvent evaporation process and measured drug release rate both *in vitro* and *in vivo*. As the drug loading increased from 16-50%, the time to 50% release ( $t_{50\%}$ ) decreased significantly *in vitro*. Decreasing the particle size also increased the rate of release. Copolymers containing 17 and 30 mole percent HV enhanced the sustained release *in vitro*. *In vivo* the sulphamethiazole plasma profile in six dogs showed that drug release was extended and uniform.

Gould *et al.* (1987) studied release rates of fluorescein isothiocyanate labeled dextrans from compressed tablets of PHB and P(HB-HV) containing the porosigens microcrystalline cellulose and lactose. At low porosigen levels drug release was matrix controlled. The release rate was also independent of solution pH except unless the aqueous solubility of the drug was itself a function of pH. In summary, the manner in which the drug is mixed with the polymer, the resulting crystallinity and porosity are important determinants of the rates and mechanisms of release.

#### 1.6.4. Biocompatibility of PHB .

Little literature is available on the biocompatibility of these

polymers. Korsatko et al. (1984) implanted tablets of PHB subcutaneously into the neck creases of mice. On histological examination an initial acute inflammatory reaction was seen and a mild inflammatory reaction continued throughout a 20 week observation period. These authors also used tissue culture techniques to determine the effect of PHB on cellular growth rate and metabolic function. The experiments indicated no significant differences in cellular growth or metabolism between control cultures and those exposed to PHB. Juni and Nakano (1987) reported on the apparent biocompatibility of PHB microspheres once implanted into the thigh muscle of rats. An initial mild inflammatory reaction was observed and after four weeks of implantation, the microspheres became encapsulated by connective tissues.

The biocompatibility of P(HB-HV) copolymers, their degradation products and the effect of different physical forms when implanted *in vivo* have not been reported. The following section describes biocompatibility testing as a whole and the rationale in the design of experiments to determine the biocompatibility of PHB, P(HB-HV) and their degradation products with reference to positive and negative controls.

## 1.7. BIOCOMPATIBILITY.

### 1.7.1 Biocompatibility and Biofunctionability - Definitions

Any new materials that become components of medical devices or drug delivery systems are required to be biofunctional and biocompatible within the cellular environment in which they are placed. Biofunctionability relates to the intended performance of the material, and ideally, the material should function as originally intended throughout the entire implantation period. Biocompatibility

is considered a property of a material and is defined as "the ability of a material to perform with an appropriate host response in a specific application" (Williams, 1988). Biocompatibility should be defined in terms of the conditions of use, (the specific application), and therefore there can be no such thing as a universally "biocompatible material". It is a term used to describe the state of affairs when a biomaterial exists in a physiological environment without either the material adversely and significantly affecting the body, or the environment of the body adversely or significantly affecting the biomaterial. These are two very broad aspects of biocompatibility but are very much interrelated, for it is often an adverse effect on a material by the physiological fluids which leads to release of particulate or soluble material from the biomaterial, which in turn leads to an adverse response from the tissues. Biocompatibility is therefore largely concerned with chemical reactions that take place between the body fluids and the implanted materials and the physiological responses to these reactions. Thus, the biocompatibility of plastics, at least in the context of soft tissue response, is to a large extent controlled by the manner in which the surrounding fluids extract residual monomers, additives and degradation products. This, perhaps, is an oversimplification of the effects of the material on the host and vice versa, as there is great complexity in these interfacial reactions.

The biocompatibility of metals and polymers may be influenced by many factors in addition to their chemistry. The mechanical relationship between implant and tissues can modify the response, which may arise either through relative movement at the interface and may influence the cellular response, or otherwise be due to the

relationship between implant and tissues can modify the response, which may arise either through relative movement at the interface and may influence the cellular response, or otherwise be due to the chemical reaction, or through the inequality of the elastic moduli which can alter the stress distribution in the tissue and consequently influence fibrogenesis and osteogenesis. The physical form of the implant has clearly been shown to be an important factor in biocompatibility in the context of the soft tissue response which includes the inflammatory response and carcinogenesis. For example, powdered material produces quite different effects to solid materials.

// be able to explain

// expand

#### 1.7.2. Criteria for biocompatibility testing.

Items falling outside the domain of drugs but which are used in medicine, dentistry and other animal or human applications which may involve contact with biological systems do not fall under any regulatory body in regard to toxicity testing programs. Therefore the safety evaluation programme is ultimately at the discretion of the manufacturer. However guidelines for biocompatibility testing have been developed and are discussed in section 1.7.4. Several kinds of reactions that would represent valid reasons for subjecting new materials to biological testing include;

- 1) irritation
- 2) inflammation
- 3) pyrogenicity
- 4) systemic toxicity
- 5) sensitization
- 6) mutagenicity
- 7) carcinogenicity



probably the most prevalent one is local tissue response. If the response is of a mild nature the body will generally overcome the toxic property imparted by the implant. Local irritancy is caused by a number of factors such as the physical form of the implant, (Matalaga *et al.*, 1976), the surgeons skill in implanting the device (Rae, 1980) bacterial contamination, (Williams, 1981), and the presence of leachable constituents. A number of biological tests, both *in vivo* and *in vitro*, have been used and still are used to determine the potential of an implant to cause a local tissue response, and these are discussed in detail in Chapters 3-6. Often the *in vitro* tests are more sensitive and the material may produce a toxic response that may not be detected when the implant is placed directly into tissue. The site of implantation is also an important factor as to the reactivity of the implants (Williams, 1973; Hench and Etheridge, 1982; van Blitterswijk *et al.*, 1985).

Systemic toxicity is an important consideration in biocompatibility testing and may occur if a constituent of the material is absorbed into the circulatory system. The extent of toxicity therefore depends on the dose reaching a particular site. Pathological changes at distant tissues and organs may be reversible, or the damage may be of sufficient intensity that the body cannot reverse the damage. In clinical situations there have been reports of death or adverse effects when methylmethacrylate cement has been used in orthopaedic practice to anchor hip prostheses to the bone. The monomers can be absorbed and will produce systemic toxicity when the level of the chemical reaches 50ml/kg of body weight (Homsey *et al.*, 1969). These low molecular weight acrylic monomers are reported to cause an initial increase in respiration followed by a fall. Hypotensive responses will occur almost immediately after the

chemical is administered to animals and the deaths of the animals have usually been ascribed to respiratory failure, even though now there is some indication that the compounds may produce cardiac arrest (Autian, 1975). Silicone coating placed on the surfaces of tubings used in extracorporeal devices has also been reported to cause the death of several patients attributable to tiny fragments of the silicone coating breaking away from the tubings and entering the circulatory system causing embolization (Lindberg *et al.*, 1961).

Allergic responses may be induced with certain chemicals used in the manufacture of biomaterials. For example, curing agents such as mercaptobenzothiazole, antioxidants such as monobenzylether of hydroquinone and phenylbetanaphthylamine (Autian, 1973). It has been reported that polymethylmethacrylate dentures caused an allergic response in some patients, (Crissey, 1965). However, reported clinical cases of allergic responses from manufactured plastic devices are quite rare. Little is known of the possible allergic responses to implants of medical devices that have long-term contact with tissue. Biodegradation of the polymer could release an agent having sensitisation potential, thereby bringing an adverse effect that may not be traced to the degradation product or even the implant.

One of the most important tests in establishing biocompatibility is that of blood compatibility, i.e. the potential of a material to be thrombogenic. The unsolved problems in the development of cardiovascular biomaterials include a definition of the basic mechanisms of blood-material interactions and its consequences and identification of predictive tests of clinical performance of materials. Many diverse materials have been used successfully in clinical and experimental cardiovascular

applications. Polyetherurethanes, carbons, Dacron, expanded Teflon and siloxanes have all been used in cardiovascular devices; polyvinylchloride is widely used for blood collection and storage. However, no single material has been defined as ideal, perhaps because these materials have been used in a variety of applications ranging from static (blood bags) to dynamic (heart valves). In the early research on circulatory assist devices, polyurethane polymers and copolymers with polydimethylsiloxane were identified as materials having potentially favourable interactions with blood. Reports have been presented demonstrating that certain silicone ball-shaped rubber heart valves absorbed lipoidal constituents from the blood and resulted in cracks or other alterations in the shape of the ball necessitating removal of the valve (Raible, 1966). Acceptable materials have not yet been identified for long term use in low-flow situations; autologous materials are still preferred as vascular grafts for small diameter vessels and coronary bypass procedures. The use of biodegradable polymers for use as vascular grafts has recently emerged and is discussed in detail in chapter 5.

One important aspect of biocompatibility is the potential of a polymeric implant to cause cancer. Cancer may occur through physical effects (solid state carcinogenesis), the presence of carcinogenic chemicals in the polymer or the development of a carcinogen during the period of implantation. A completely new biomaterial must be evaluated for its possible carcinogenic properties. Recently certain *in vitro* mutagenicity tests, using specific bacterial lines, such as those proposed by Ames (1975), have been used as rapid methods of ascertaining the carcinogenic potential of these biomaterials. However there is much controversy in the literature of toxicology and safety evaluation as to the most satisfactory model and design of

animal study to predict the carcinogenic activity of a polymer in humans. The debate also continues as to the appropriateness of using the subcutaneous tissues of rats for the testing of solid materials such as plastics, because it is recognised that rat subcutaneous tissues are much more sensitive than human with regard to the development of tumours. The use of dogs or subhuman primates for testing the carcinogenic potential of polymers is also precluded as the latent period for development of tumours is several fold longer than the 6 month to a year period observed in rodents. Testing for the carcinogenic activity of polymer implants often follows the acute toxicity screening programme, once a new biomaterial is considered to be biocompatible for its specific application. However such testing was beyond the scope of this work due to the long periods required for completion of such tests.

#### 1.7.3. Development of toxicity programmes.

Dixon and Rickert (1933) published a paper on tissue response to foreign materials. Dental materials were implanted subcutaneously and intra-muscularly in rabbits and, over a 6 month period, the animals were killed and the tissue surrounding the implant was prepared for histopathological examination. The above authors were among the first to develop a histopathological scoring system for tissue response for use in this type of toxicity testing. In the late 1950's, with an increase in the number and variety of disposable plastic devices used (collection and storage devices for drugs, biological fluids and diagnostic products) Brewer and Bryant (1960) utilized an intramuscular implantation test to determine their safety. This consisted of preparing the samples as thin rods and introducing them by way of a hypodermic needle into the paravertebral

muscles of rabbits. After three days the animals were killed and the sites examined for gross tissue response. Any samples which revealed tissue responses were considered to fail the test. Lawrence *et al.* (1963) in their study of a number of polyvinyl chloride administrative devices, utilised the method of Brewer and Bryant, but extended their implantation period to 7 days. The method of Brewer and Bryant was adopted in 1965 by the United States Pharmacopeia as one of the biological tests for testing plastics to be used for containers and items having contact with parental products. The official test requires the use of a polyethylene negative control.

Powell *et al.* (1970) devised a toxicity evaluation programme for dental materials. These authors employed a tissue culture test, intramuscular and intradermal administration to rabbits and also dermal and ocular tests in rabbits. The latter two tests were concluded not to be suitable experiments.

Turner and associates (1973) studied the subacute tissue response of a number of medical and dental items using essentially the same USP implantation test as that described above. Both gross observations and histopathological examinations of tissue are the prominent means of evaluating the response of implants. In 1976 the Materials Science Toxicology Laboratories developed a Primary Acute Toxicity Screening Programme for new biomaterials. In 1977, Autian published the test procedures. The biological responses were graded according to the intensity and all of the scores ranging from 0 (no response in any test) to 1500 (most intense response for every test) were then compiled into the Cumulative Toxicity Index (CTI). Materials having CTIs below 100 were considered good candidates for further consideration for biomedical applications. Greater sensitivity of biocompatibility testing can be achieved by the use of

histochemical methods and by examining the fixed tissue by electron microscopy (Salthouse *et al.*, 1976). The use of enzyme histochemical techniques for assessment of the biocompatibility of a new material is described in detail in chapter 6 in relation to the *in vivo* assessment of PHB, P(HB-HV) and their degradation products.

Other workers in the field of biomaterials have established specific techniques for the assessment of biocompatibility specific to the material's application these techniques are considered in detail in subsequent chapters.

#### 1.7.4. Present guidelines for safety and toxicity testing of biomaterials.

In the last decade, a number of countries and organisations have developed general guidelines for the testing of toxicity and safety of medical materials and devices. In the United States a number of biological testing programmes for safety of new biomaterials and devices have evolved. As early as 1965, the United States Pharmacopeia (USP) included a section on the biological testing of polymers to be used with parenteral products. The tests included implantation of the polymer into rabbit muscle tissue with subsequent biological tests to be performed on extracts (using four extracting media). Extracts of the polymer are also assessed using an intracutaneous test in rabbits for irritancy and in a systemic toxicity test in mice.

Each year the guidelines are revised. Presently the preferred animal model is the rabbit and the implantation test involves the preparation of 10 x 1mm diameter sterile test strips, which are implanted by means of a hypodermic needle. Four strips of a control (designated the USP Negative Control Plastic RS) are used. Four

strips of the test material are implanted on one side of the spine in each of two rabbits , 2.5 to 5cm from the mid-line and parallel to the spinal column. Two strips of the control are similarly implanted into the opposing muscle. The animals are sacrificed 72 hours after implantation and the tissue around the centre portion of each implant is examined microscopically with attention given to the presence or absence of haemorrhage and encapsulation.

The Federation Dentaire International (1980) published recommended standard practices for the biological evaluation of dental products including a sub-cutaneous (s.c.) implantation test. In the F.D.I. recommended practice, guinea pigs are specified, although other animals may be used. Its tests are rigorous in detail and involve the examination of the s.c. tissue for the presence of necrosis, inflammation and the resorption of material at two and twelve weeks. A specific scale of toxicity is given to the materials according to the degree of inflammation.

The American Standards for Testing and Materials (ASTM) have also published a number of standard practices for biocompatibility testing. The rabbit is the recommended animal, but the rat or other species are allowed. Implantation procedures are very similar to those specified in the USP. Gross observations are recorded at 7 days and histopathology is performed at 7, 30, and 90 days. If the performance of the polymer in this initial short term test is promising, the biomaterial is tested on a longer term basis with histopathology carried out at 2, 3, 8, 12, 52, and 104 weeks in the rat.

In Great Britain the British Standards Institution provides a set of recommendations for toxicity tests with a defined set of acceptable and nonacceptable criteria. In the implantation test New

Zealand rabbits are specified, into which two positive and two negative controls and four test specimens are implanted. The implants are left in place for 2 days before the animal is killed. Macroscopic observations and then histological preparations are carried out. The assessment of results are in subjective in that a reaction is considered negative either if there is no reaction or if there is a reaction that can be attributed to experimental trauma (typically asymmetrical, nonnecrotic and noninflammatory) and positive if there is necrosis and/or inflammation symetrically around the implant.

Many workers believe the above tests are too simplistic in nature and very subjective to individual interpretation. More astringent biocompatibility testing is required to give quantitative data.



### 1.8. Scope of Present Study.

The work presented in the following chapters studies the biocompatibility of PHB, P(HB-HV) and their degradation products utilising both *in vivo* and *in vitro* techniques. Acute and chronic aspects of the implant-host interactions have been evaluated including vascular permeability changes; inflammatory response in terms of cellular infiltration, assessed by microscopy and enzyme histochemical techniques; macrophage activation and blood compatibility.

One possible application of these polymers, controlled drug delivery, has been assessed both *in vivo* and *in vitro*, in terms of biocompatibility and the kinetics of drug release.

## CHAPTER 2. Materials and Methods.

This chapter has been divided into 4 sections for convenience.

Sections 2.1-2.3 describe *in vitro* techniques used to assess polymer and monomer biocompatibility and Section 2.4 describes *in vivo* techniques.

2.1 Cell culture.

2.2 Effect on macrophage population (activation/inhibition).

2.3 Protein/platelet adsorption.

2.4 *In vivo* assessment of the soft tissue inflammatory response.

## 2.1. Cell culture: Materials and Methods

### 2.1.1. Equipment

Laminar flow hood; 2m, horizontal displacement type; Fell Clean Air (1971) Ltd., Newhaven, Sussex.

The surfaces of the hood were swabbed with 70% ethanol before and after use.

Incubator; LEEC PF<sub>2</sub> anhydric incubator with forced air circulation, Laboratory and Electrical Engineering Co, Nottingham.

The thermostatic controls were adjusted to maintain a temperature of 37.5°C +/-1°C.

Centrifuge; Beckman bench centrifuge model no. TJ-6; Beckman Industrial Estate Mervue, Galway, Ireland.

Adjustable replicating pipettes;

Pipette	Effective volume range
Gilson pipetman P20	2-20ul
Gilson pipetman P200	20-200ul
Gilson pipetman P1000	200-1000ul
Gilson pipetman P5000	1000-5000ul

Pipettes with disposable polypropylene tips were obtained from Anachem Ltd., Luton, Beds. Pipettes were periodically checked to ensure accurate and reproducible sample delivery.

Liquid Nitrogen Freezer; Union Carbide model No. LR-33-10; Union Carbide UK Ltd., Cleveland, in which ampoules containing the stock cultures were held in the liquid N<sub>2</sub> refrigerant or in its overlying

vapour.

Freezing unit; Union Carbide BF-6 biological freezer, a plug type device designed for use with the LR-33-10 freezer. This unit is capable of cooling eight 2ml ampoules to below  $-70^{\circ}\text{C}$  at a cooling rate of between  $0.5^{\circ}\text{C}$  and  $7^{\circ}\text{C}$  per minute.

Haemocytometer; standard double grid improved Neubauer-type blood cell haemocytometer with coverslips; Fisons Ltd., Loughborough, Leicester.

Microscopes;

1) For examination of growing cell cultures under phase contrast an inverted biological microscope, Wild M40, Wild Heerberg Ltd., Heerberg, Switzerland, was used. This fitted with the appropriate condensers and x10 and x20 objectives gave magnifications of x187 and x375 respectively.

2) For haemocytometer and other cytological preparations an Amplivial microscope, Carl Zeiss Jena, CZ Scientific Instruments Ltd., Boreham Wood, Herts, was used. With fitted condenser and both plain and phase-ringed plane field objectives (x16, x40, x100) this instrument allowed transmitted light and phase contrast microscopy at magnifications of x256, x640 and x1600 respectively.

Disposable cell culture plasticware; Tissue culture (T/C) grade disposable  $25\text{cm}^2$  plasticware was obtained pre-sterilized from Flow Laboratories Ltd., Rickmansworth, Herts. 90mm T/C petri-dishes with triple vents were also obtained from Flow Laboratories. 2ml polypropylene screw-capped ampoules for liquid nitrogen cell storage

were obtained pre-sterilized from Sterilin Ltd., Feltham.

Glassware; 100ml and 500ml bottles for the storage of solutions and media were obtained with caps from Flow Laboratories Ltd, Herts.

150ml and 500ml soda glass 'medical' flat bottles were obtained from Fisons Ltd.

#### 2.1.2. Cell Line

CHO-K1; a L-Proline requiring Chinese Hamster Cell line (Kao and Puck 1968) were cultured and characterized at Bath by R.S. Dewdney (1982). These cells were used in experiments between passages 30 and 40.

##### History of CHO-KI cell line

CHO-K1 is a well characterized cell line established as American Type Culture Collection (ATCC) Certified Cell Line (CCL) 61. It was derived as a subclone from the parental CHO line by Kao and Puck (1968) and shares most of its properties. CHO itself was one of a series of clones isolated from a culture initiated in 1957 from a biopsy of Chinese Hamster ovary (Puck et al 1958). CHO cells were initially fibroblast-like but have undergone several hundred passages since then and the cells of the K1 subclone more closely resemble epithelial cells. CHO-K1 is hardy, reliable and routinely grown at 37.5°C as monolayers on glass or plastic culture flasks in nutrient media containing relatively low levels of Foetal Calf Serum, (5%v/v) Under optimum conditions the cells grow rapidly with a population doubling time of less than 16 hours and show high single-cell plating efficiencies, (Raetz et al., 1982). These characteristics coupled with the ability to grow easily in suspension, aswell as on solid surfaces, are advantageous in many types of experiment.

### 2.1.3. Cell Culture Materials

Water; Double glass distilled water (DDH<sub>2</sub>O) was used in the preparation of all solutions and media. This was produced by a 6KW, 4.5 litre per hr bi-distillation Fi-stream still, model 2903 fitted with a Fi-stream pre-ionizer, Fisons Ltd, Loughborough. DDH<sub>2</sub>O was sterilized by autoclaving at 121°C for 15 minutes in 100 and 500ml volumes.

Phosphate Buffer Saline (PBS) : Formula [g.l<sup>-1</sup> in DDH<sub>2</sub>O]- sterilized by autoclaving for 30 minutes at 115.5°C.

NaCl	8
KCl	0.2
Na <sub>2</sub> HPO <sub>4</sub>	1.15
KH <sub>2</sub> PO <sub>4</sub>	0.20
*CaCl <sub>2</sub> .2H <sub>2</sub> O	0.132
*MgCl <sub>2</sub> .6H <sub>2</sub> O	0.10

\*A buffer was also used in the absence of these salts and is referred to as PBSA.

Trypsin solution: A filter sterilized 2.5% w/v solution of trypsin obtained from Flow Laboratories. This was diluted to a working strength of 0.025%w/v in the presence of 0.02% EDTA in PBSA and stored as 8ml volumes in 10ml glass bijoux bottles at -20°C.

Additives for cell culture media: Antibiotic solution:

Penicillin(5,000iu ml<sup>-1</sup>) and streptomycin (5,000micro-grammes ml<sup>-1</sup>) solution obtained in 100ml unit quantities from Flow Laboratories.

200mM L-Glutamine (Flow Laboratories)

100mM L-Proline (Flow Laboratories)

Sodium bicarbonate: obtained as a sterile 7.5%w/v solution from Flow Laboratories and stored at room temperature.

Media: Modified Ham's F10 medium (MF10)

Prepared aseptically:-

428ml sterilized DDH<sub>2</sub>O

50ml 10x concentrate of Ham's F10 medium (without L-Glutamine and NaHCO<sub>3</sub> component) from Flow Laboratories

5ml Penicillin (5,000IU ml<sup>-1</sup>) and streptomycin (5,000micro-grammes ml<sup>-1</sup>) solution.

1ml 100mM L-Proline

7.5ml 200mM L-Glutamine

8ml 7.5% w/v NaHCO<sub>3</sub>

Stored in 100ml volumes at 4°C and used within 4 weeks.

Sera: All culture media were supplemented with 5% v/v FCS. (MF10V) FCS from two different batches was used during the course of this study. These had previously been tested for their ability, as a supplement to culture medium, to satisfactorily support the clonal growth of relevant mammalian cells.

Batch 1 BN: 601109 Sera-Labs Ltd, Crawley, Sussex.

Batch 2 BN: 601103 Sera-Labs Ltd, Crawley, Sussex.

The sera was stored frozen at -20 °C until use.

Cryoprotectants: Glycerol: Analar grade (Fisons Ltd) stored at room temperature protected from moisture. 2-3ml volumes were sealed in glass ampoules and sterilized by dry heat (160°C for 1 hour).

Biological Stains: Methylene blue (BDH Chemicals) was prepared as a 5% w/v solution in 50%v/v methanol. It was used to stain mammalian cell colonies attached to tissue culture plates.

Replica plating materials:

Nunclon plastic tissue culture petri dishes, 90mm diameter, Roskilde, Denmark. Polyester mesh; 17um Polymon mesh, Plastok Associates Ltd, Birkenhead. Circles of mesh were cut using a 85mm diameter template, with a notch at the edge to facilitate matching of replicas. Glass ballotini; 4.5-5.5mm in diameter, were obtained from BDH Chemicals Ltd, Poole, England.

## General Methods

### 2.1.4. Gases and Gassing Procedures

Gases:

Cylinders of N<sub>2</sub>, CO<sub>2</sub> and 5% CO<sub>2</sub> in air mixture (5% CO<sub>2</sub> and 20% O<sub>2</sub> in N<sub>2</sub>) were obtained from Air Products Limited, Bristol Gas flowmeter: Floating needle type, 0.1-1.0 min<sup>-1</sup> (Rotameter Manufacturing Co. Ltd., Croyden.) calibrated for use with CO<sub>2</sub>. Plastic incubation boxes: Rigid, clear plastic boxes, 3.25 litre volume (A.Gallenkamp and Co Ltd, Finsbury Square, London) Gas-tight tape: British Standard gas-tight tape, 2.5cm wide (Intech Tapes Ltd, Manchester.)

All cell culture media contained a bicarbonate pH buffer system designed to equilibrate with an atmosphere of 5% CO<sub>2</sub> in air. All air-tight culture bottles and flasks were therefore charged with such a gas mixture, introduced at low flow rate through a sterilized Pasteur pipette plugged with non-absorbent cotton wool. Cell culture



dishes and other non-sealable vessels were placed in plastic incubation boxes into which a metered volume (150ml) of pure CO<sub>2</sub> was introduced through a plugged pipette. These boxes were sealed with gas-tight tape. If only a few plates were put into a particular box, an open 9cm petri dish filled with water was added, to prevent excessive evaporation from culture medium during incubation.

#### Cell Culture Methods

2.1.5. Maintenance of cell line: CHO-K1 is capable of growth on glass surface and thus stock cultures were routinely maintained in MF10V medium in 125ml clear soda-glass "medical flat" bottles at 37.5°C. Under these conditions cells grew as a monolayer on the bottom surface of each horizontally placed bottle. Cultures were not allowed to reach the point where release of cells from a confluent monolayer began since this can lead to increased heterogeneity within cell populations (Ham and Puck 1962). The routine subculture protocol was designed so that cells remained at all times in a condition of active growth and never achieved full confluence.

2.1.6. Preparation of cell suspensions from monolayer cultures: The essential manipulation involved in maintaining the CHO-K1 cell line was subculture. This operation required that cells be obtained in suspension prior to transfer to fresh culture vessels. Cells once in suspension could also be greatly diluted and plated so that colonies arose from single cells, a procedure fundamental to many experiments.

For a routine sub-culture the medium had to be clear with no floating cellular debris and not unduly acidic or basic, as colour indicated by its phenol red component. Cell growth was required to be greater than 50% confluent and the cells were required to have a normal appearance i.e. normal epithelial morphology.

The medium was removed from the culture and the cells rinsed gently with 5ml of 0.05% trypsin solution. This solution was then discarded and another 1ml of trypsin added. The culture was incubated for about 5 minutes at 37.5°C and then shaken vigorously. Detachment of cells was checked microscopically. The cells were gently aspirated with a Pasteur pipette, to break up clumps and produce a uniform suspension and then added to 5ml of complete medium in a 16mm T/C tube. Though the serum and  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions in the medium protected the cells against continued deleterious enzyme action, the carried over trypsin was further diluted as soon as possible to minimise any risk of cell damage. Subculture or experimental manipulation of cells was therefore carried out immediately after trypsinisation.

2.1.7 Determination of cell suspension density: This was achieved by means of a haemocytometer count. Cells were thoroughly mixed immediately before sampling. A small volume was withdrawn from just below the surface of the suspension and introduced into the haemocytometer chamber. A total cell count was performed on eight large squares of the haemocytometer grid under low power (x256) phase optics. If the cell density was greater than 150 per large square, at which point cell counting was impeded by crowding, the suspension was diluted with medium and sampling and counting repeated. In the event of a very low density (less than 25 per square), the cells were concentrated by centrifugation, resuspended in a smaller volume and resampled.

The large squares of the haemocytometer had an area of  $1\text{mm}^2$  and when the coverslip was pressed down over the grid, the depth of the chamber was  $0.1\text{mm}^2$ . The total volume over each large square was

therefore:

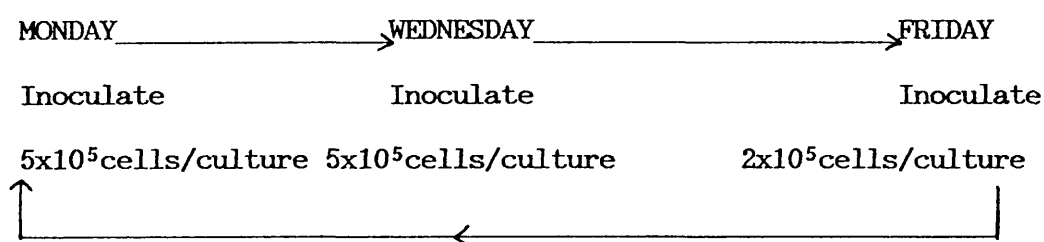
$$1 \times 1 \times 0.1 = 0.1\text{mm}^3 = 0.0001\text{cm}^3 = 10^{-4}\text{ml}.$$

The total cell count per ml i.e. the cell density, was given by :

cell  $\text{ml}^{-1} = 10^4 n$  where  $n$  was the the average number of cells per large square.

#### 2.1.8. Subculture routine:

Cells were subcultured at low inoculation density three times per week using the following protocol:



Fresh cultures had to be initiated with small inocula not only because cells were to be maintained in vigorous growth, but also because F10 medium, which was designed to support the clonal growth of CHO cells is not particularly well suited to supporting dense populations (Ham 1965). Aliquots of counted cell suspension were added to 14.5ml volumes of MF10V in standard 125ml culture vessels and evenly distributed by gentle aspiration followed by agitation.

Ham's F10 contains a bicarbonate pH buffer system designed to equilibrate with a 5% CO<sub>2</sub> in air atmosphere (Ham 1963), therefore such a gas phase had to be provided in all cultures. The culture bottles were purged with the said gas mixture for 10-15 seconds and capped air-tight, prior to incubation. Bottles were incubated flat for maximum surface area for growth. Incubation, at 37°C, was in the dark because cytotoxic photoproducts are known to form from certain

components of mammalian cell culture media (Wang et al. 1974).

#### 2.1.9. Cell storage:

$5 \times 10^5$  cells were routinely stored in the presence of cryoprotectant, 8% glycerol, in liquid N<sub>2</sub> (at -196°C).

2.1.10. Cell recovery from storage: After removal from the liquid N<sub>2</sub> refrigerator the contents of an ampoule were rapidly thawed at 37°C. The cells were transferred by Pasteur pipette to 14ml of MF10V in a culture bottle and gently mixed. The bottle was gassed with 5% CO<sub>2</sub> and incubated. Invariably, within a couple of hours cells attached to the bottom glass surface and after three days had grown sufficiently to be passaged and subsequently handled as a routine stock culture.

#### 2.1.11. Dilution Plating:

The plating of a small number of single cells into T/C petri-dishes, and their subsequent growth into macroscopic colonies, was fundamental to the replica plating and colony formation assays carried out. The following protocol was the basis for all dilution plating procedures. 4.9ml aliquots of culture medium in 50 mm T/C petri-dishes were equilibrated in plastic incubation boxes at 37.5°C in a humidified 5% CO<sub>2</sub> in air atmosphere. Attachment of cells can occur rapidly at 37°C (Ham and Puck, 1963) and therefore to prevent cell loss all other media and solution were used at room temperature. A cell suspension was prepared and the cell density determined, the counted cell suspension being serially diluted to a final density of  $10^3$  viable cells ml<sup>-1</sup>. Replicate 0.1ml volumes of the final dilution were added to the equilibrated T/C dishes and the cells evenly distributed by gentle circular agitation. Inoculated dishes were

replaced in the incubation boxes with a 5% CO<sub>2</sub> in air atmosphere. The boxes were sealed with gas-tight tape and incubated in darkness at 37.5°C for 7-8 days. During incubation the dishes were left undisturbed to prevent the formation of erroneous satellite colonies.

2.1.12. Staining: To determine the plating efficiency of the cells, the medium was carefully poured off from the plates and then each flooded with 5% methylene blue in 50%v/v methanol. After 30 minutes the dishes were rinsed under running tap water and allowed to dry. Stained colonies were scored by the naked eye against a white background, with aggregates containing 50 or more cells qualifying as a surviving colony. Colonies of marginal size were examined under a binocular dissecting microscope to confirm that they fulfilled this criterion.

2.1.13. Determination of the growth parameters of CHO-K1 cells.

Inocula of  $2 \times 10^5$  CHO-K1 cells at passage 32 were subcultured with 5ml MF10V into a number of 25cm<sup>3</sup> T/C flasks and incubated at 37.5°C. At intervals a culture was removed and its total cell number determined as follows.

The medium in the culture flask was withdrawn and placed in a 16mm, graduated, V-bottomed test tube. The cell monolayer was washed with 2ml of trypsin solution and then disaggregated in 1ml of trypsin. Both wash suspension and suspended cells were added to the medium in the tube. To remove residual cells the flask was rinsed successively with two 1ml volumes of PBS. Removal of all the cells was confirmed by microscopic examination. To minimize cell loss due to adherence to glass surfaces, siliconized Pasteur pipettes were used for all above transferences. Cells were sedimented by

centrifugation and resuspended in about 0.5ml of supernatant. The suspension was adjusted to an expected cell density of  $2.5 \times 10^5$ – $1.5 \times 10^6$  cells per ml with PBS and the volume of this final cell suspension noted. The cell density was determined by haemocytometer counting a minimum of 400 cells, and the total cell number of the culture calculated.

CHO-K1 cell lines exhibit 'classical' growth kinetics, growth being divided into three distinct phases: (Figure 2.1.)

1) Lag phase: this is the time following sub-culture and seeding where there is little evidence of an increase in cell number. It is a period during which the cell replaces elements of the glycocalyx lost during trypsinisation, attaches to the substrate and spreads out (Freshney, 1983).

2) Log phase: this is the period during which cell numbers increase exponentially. It follows lag phase and is also known as the exponential growth phase.

3) Stationary phase: towards the end of the log phase the culture becomes confluent i.e. all the available growth surface is occupied and the cells are in contact with neighbouring cells, hence a reduction in cell growth rate follows (Freshney, 1983). Figure 2.1 shows a typical growth curve of CHO-K1.

From such growth curves cultures were determined to have a lag time of 6–8 hours. After approximately 9–12 hours incubation time cell growth was exponential and could be represented by the equation:

$$N_t = N_0 2^{kt}$$

where  $N_0$  is the cell number at time 0,  $N_t$  is the cell number at time  $t$  and  $k$  is the exponential growth rate constant. The calculated value of  $k$  ( $\text{hr}^{-1}$ ) from the slope in figure 2.1 was 0.089 and therefore the population doubling time (the inverse of  $k$ ) was 11.2 hours.

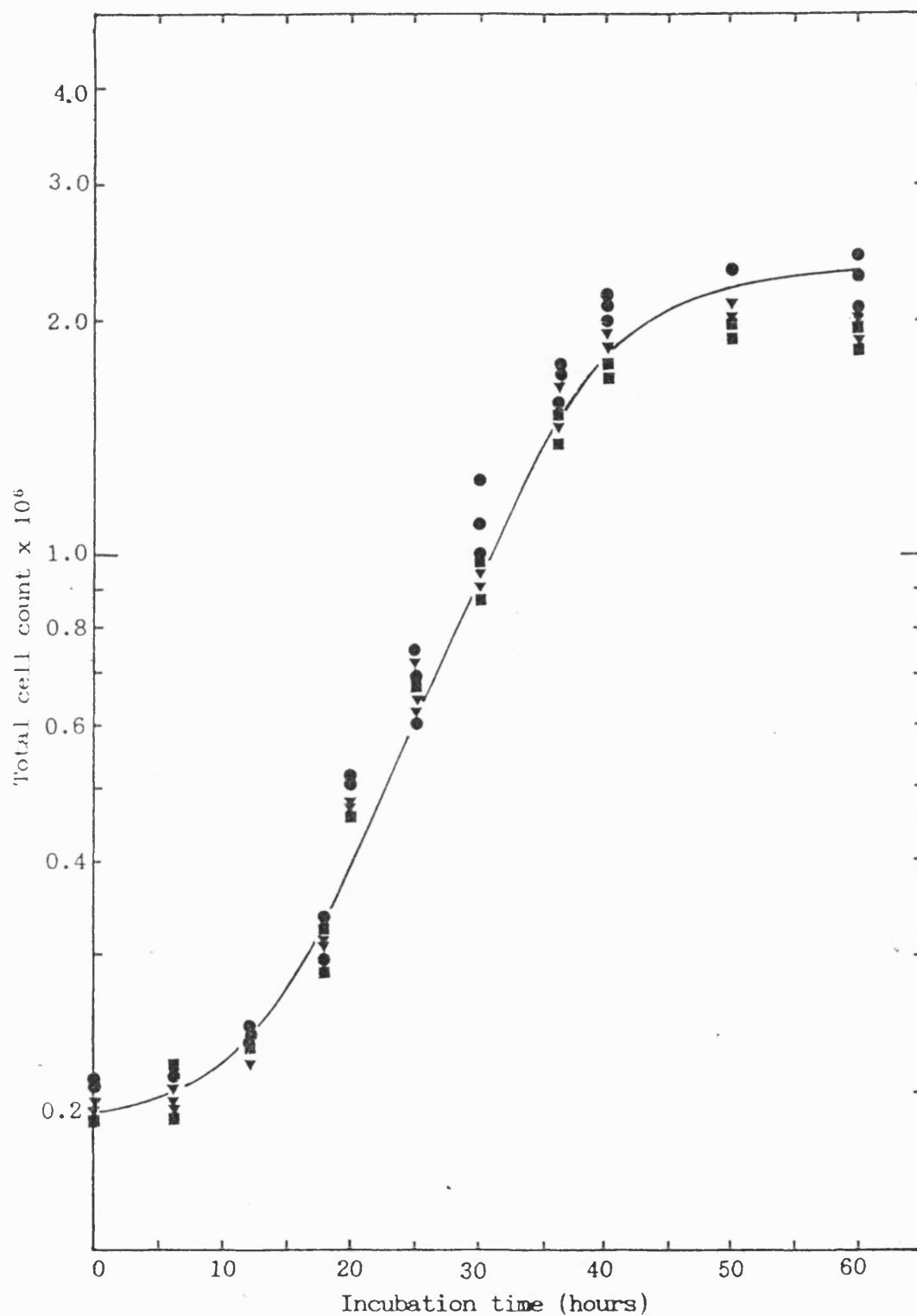


Figure 2.1. Growth curve of CHO-K1 cells grown in Hams F10 + 5% FCS (■, ▼, ●). Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.

Exponential growth ended after approximately 40 hours and the cells proceeded to stationary phase by 60 hours.

#### 2.1.14. Construction of growth curves

Strips of polymer films (3cm x 1cm) were cut and sterilized in an autoclave at 121°C for 15 minutes. Four strips were then suspended in 25cm<sup>2</sup> tissue culture flasks containing 5ml of modified Ham's F10 culture medium (MF10V) with a sodium bicarbonate buffering system to ensure a pH of 7.2 - 7.6. The flasks were then inoculated with  $2 \times 10^5$  cells per flask and growth curves constructed over a 60 hr period. In a separate experiment the cells were left in contact with the PHB or P(HB-HV) films for 48 hrs, reharvested into control medium and their subsequent growth followed. The degradation products, HB and HV as their sodium salts, were dissolved in the buffered media at concentrations of 1,3,5,10,15,20,25 and 35mg/ml and inoculated with  $2 \times 10^5$  cells for the study of growth curves. The lag and population doubling times were then calculated and these parameters compared with those of untreated cultures. As a positive control for the effect of polymers on cell growth, polyacrylic acid (PAA), which is known to be cytotoxic was used in crosslinked gel form. Polymethylmethacrylate, (PMMA), a known non-cytotoxic material was used as the negative control.



### 2.1.15. Replica Plating

#### Introduction

The technique of replica plating is used commonly when working with bacteria and yeast but is less easily applied to mammalian cells. The principle of replica plating involves the inoculation of low densities of single cells into petri dishes allowing the development of macroscopic colonies. The colonies are then "copied" onto e.g. polyester mesh (Raetz *et al*, 1982) overlaid with the chemical under test, and after specified (hours) exposure to these materials, the viable colonies stained with methylene blue. The corresponding positions on each replica plate can then be located and compared with the master plate.

#### Procedures

##### A. Preparation of materials.

Systematic preparation of both polyester and glass balls was essential for consistent results. Cleaning and sterilization were carried out before the first use; and both the glass balls and polyester could be used repeatedly after a recycling treatment. Initial preparation and recycling of glass balls: The balls were soaked overnight in 10%(w/v) nitric acid, then washed thoroughly in cold running water (2hr). They were subsequently soaked for two hours in sodium hypochlorite solution (12% available chlorine), diluted 1 in 50 with water, then again washed thoroughly in running water. The glass balls were boiled in detergent (RBS) for 5 minutes, washed for several hours in running water, and then rinsed with four changes of distilled water, draining well each time. They were placed into a beaker, covered with foil, and sterilized by autoclaving.

Initial preparation and recycling of polyester : A batch of polyester

circles (85mm diam. with notch) were soaked in dilute sodium hypochlorite solution (12% available chlorine) for about 30 minutes then washed in running water for 1-2 hours. The polyester was boiled for 5 minutes in dilute RBS detergent and washed overnight in running water. The circles were then rinsed with four changes of distilled water and drained. Finally the circles were placed in stacks in a 90mm petri dish containing 95% ethanol so that all circles were immersed for 30 minutes. The ethanol was drained off and the sterilized circles were then ready for use.

#### B. Initial plating of the cells

The cells were prepared in single-cell suspension (normally by trypsinisation from the stock culture), and 100 added to the growth medium, and inoculated into 90mm tissue culture dishes.

#### C. Setting up primary replicas

After one week the medium was changed and the sterile polyester circles added (minimum of three) with the notches aligned. Sterile glass balls were gently tipped onto the polyester and spread with a sterile spatula to produce a complete single layer. The underside of the dish was marked at the position of the notch and the dishes incubated for one week at 37°C. The balls were then tipped off and 5ml of warm PBS added to each dish. Each polyester circle was transferred to a new dish containing 7ml of growth medium. Colonies were now visible as opaque dots on the translucent mesh and once the colonies were established on each replica disc the test polymer circles, PHB, P(HB-HV), PMMA and PAA were laid over the polyester mesh and the culture incubated for 24,48 and 72 hrs. The effects on the growth of established colonies were then observed following

staining of the colonies with methylene blue and the results compared with a control polyester disc, i.e the master copy of the replica plates.

#### 2.1.16. Effect of osmolarity and pH

The osmolarity of the media containing the monomers as their sodium salts was measured using a Camlab osmometer (Roebbling). pH was determined using a combined pH electrode attached to an electronic pH meter (Corning). Two substances, NaCl and sucrose, in concentrations which gave similar pH and osmolarity readings to the monomer solutions were used as comparative indexes in the cellular toxicity testing of monomer solutions.

## 2.2. Assessment of Macrophage activation : Materials and Methods.

### 2.2.1. Materials

#### General materials:-

Sterilized disposable 5, 10 and 20ml syringes; Plastipak, Beckton Dickinson, Leicester.

Sterile disposabable 20 and 25 gauge needles; Plastipak, Beckton Dickinson, Leicester.

Sterile 15ml conical centrifuge tubes; Flow Laboratories, Herts.

Sterile plugged pasteur pipettes; Richardsons, Leicester.

Sterile scissors and forceps.

Haemocytometer; standard double grid improved Neubauer-type, Fisons Ltd, Loughborough.

Sterile 24-well multidish; Flow Laboratories, Herts.

Giesma stain; Sigma Chemicals Co, Poole, Dorset.

#### Culture media:-

Heat inactivated foetal calf serum (heated at 56°C for 30 minutes), BN 61109; Flow Laboratories, Herts.

Peritoneal lavage medium :Hank's Balanced salts; Flow Laboratories, Herts.

Macrophage culture medium :RPMI X1640 Medium 1X with 20mM Hepes buffer without sodium bicarbonate and L-Gluatamine; Flow Laboratories, Herts.

#### Test materials:-

Phosphate Buffered Saline (PBS) pH 7.3; Dulbecco's, Oxoid Ltd, England.

Powdered chalk ( $\text{CaCO}_3$ ), Aldrich, Gillingham, Dorset.

PHB Mw 43,000, Marlborough Biopolymers; Teeside.

P(HB-HV) 12.6 mole% Mw 760,000, Marlborough Biopolymers; Teeside

Polymethylmethacrylate; Aldrich Chemicals Ltd, Dorset.

Polyacrylic acid; Aldrich Chemicals Ltd, Dorset.

Thioglycollate broth; Difco laboratories, Surrey.

Zymosan (opsonised), Sigma Chemical Co, Poole, Dorset.

HB as the sodium salt, Sigma Chemicals Co, Poole, Dorset.

HV as the sodium salt, Sigma Chemicals Co, Poole, Dorset.

All test materials either as solutions or suspensions in PBS were sterilized at 121 °C for 15 minutes prior to use and tested for sterility.

#### Assay kits:-

Prostaglandin E2 radioimmunoassay kit, Amersham, Bucks.

Lactate Dehydrogenase diagnostic kit, Sigma Chemicals Co, Poole, Dorset.

#### Animals:-

Six to eight week old female mice of the CLFP strain, fed on labure rat pellets and water ad libitum.

#### Methods

##### 2.2.2. Production and collection of macrophages.

The kinetics and quantification of macrophage accumulation was studied using the method below. Six to eight week old female CLFP mice were injected with 0.5ml of the test material intraperitoneally, using a 20 gauge needle. Four days later the mice were killed by CO<sub>2</sub> asphyxiation, their abdomens swabbed with 70% alcohol and 5ml of cold sterile Hank's balanced salts containing 10U of heparin per ml, were injected into the peritoneal cavity. The

abdomen was massaged gently for 1-2 minutes, a 2cm incision was made in the abdominal wall and the peritoneal exudate collected with a pasteur pipette into conical centrifuge tubes. The cells obtained were harvested by centrifugation at 1500rpm (4°C) for 10 minutes and the supernatant discarded. The cell pellet was dispersed in 5ml of RPMI X1640 culture media. The cells were counted using a haemocytometer and assessed for viability by phase contrast microscopy. The macrophage population as a % of the total cell number was determined using a Giesma stain. Cell exudates were sometimes found to be contaminated with blood probably as a result of mechanical damage to internal tissues or organs during i.p. injection or cell collection. Exudates of this nature were discarded since the presence of blood leukocytes interfered with the quantification of peritoneal macrophage accumulation.

#### 2.2.3. Macrophage cultures

Peritoneal macrophages were suspended at a concentration of  $2 \times 10^6$  macrophages per ml in the culture medium. 1ml aliquots of this suspension were then plated into each well of a 24-well microtitre plate. The cells were incubated for three hours at 37°C during which time the macrophages adhered to the plastic substrate. Non-adherent cells were subsequently removed by gentle aspiration of the medium, twice washing the plates with fresh culture medium supplemented with 10% heat inactivated foetal calf serum to maintain the macrophage cultures in vitro. The cultures were incubated for 24 hours at 37°C in an atmosphere of 95% air- 5% CO<sub>2</sub> prior to treatment with test materials and assessment of their phagocytic activity.

#### 2.2.4. Coverslip cultures

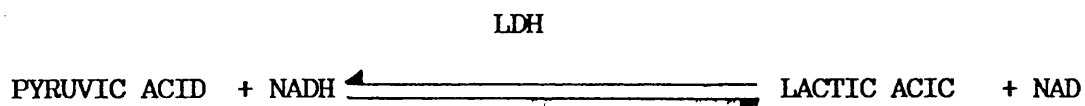
Peritoneal macrophages elicited using 0.5ml PBS injected 4 days prior to culture, were suspended at a concentration of  $2 \times 10^6$  cells per ml in the culture medium. Sterile (12mm diam.) glass coverslips were placed aseptically into the wells of 24-place microtitre plates and 0.5ml of the macrophage suspension seeded onto the surface of each coverslip. The cultures were incubated for 3 hours at 37°C in 95% air -5% CO<sub>2</sub> mixture to allow the macrophages to adhere to the glass substrate and non-adherent leukocytes removed by washing each well with culture medium. The test and control-treated coverslip cultures were then supplied with 1ml of fresh medium containing the test materials or medium alone as appropriate. These coverslip cultures were incubated as above for 15 hours, the medium removed and the cells fixed with 2.5% gluteraldehyde in Hank's Balanced Salts before assessment of their appearance by scanning electron microscopy.

#### 2.2.5. Macrophage activity assays

PGE<sub>2</sub> assay:- After the macrophage cultures had been incubated for 24 hrs the culture medium was removed and replaced with suspensions or solutions of the test materials in fresh media. The concentration of PGE<sub>2</sub> in the test or control treated cultures was assayed at intervals (0-30mins, 1-24hr) using a radioimmunoassay kit (Amersham UK procedure no. TRK 800).

#### 2.2.6. Cell viability assay

Lactate dehydrogenase activity (LDH) was assayed in each control and test culture using a Sigma colourimetric assay kit (Procedure no. 500) based on the following reaction :



The reaction equilibrium strongly favours reduction of pyruvate to lactate at a rate proportional to the amount of LDH. Pyruvic acid reacts with 2,4 dinitrophenylhydrazine to form an intensely coloured hydrazine which has peak absorbance at 450nm.

#### 2.2.7. Scanning Electron Microscopy

Fixed macrophage cultures were rinsed in PBS (pH 7.4) and the cells post fixed in a 1% osmium tetroxide solution at room temperature for 30 minutes. The specimens were again rinsed in PBS and dehydrated in an ascending series of alcohols (30 -100%), then critical point dried (Polaron Equipment, Watford, Herts.) The coverslips were then sputter coated with gold using a Edwards Pirani 1D sputter coater S150B and observed with a scanning electron microscope, Jeol 330, U.S.A.



## Assessment of blood compatibility : Materials and Methods.

### 2.3.1. Materials.

Bovine Serum Albumin; Sigma Chemicals Co, Poole, Dorset.

Bovine Fibrinogen; Sigma Chemicals Co, Poole, Dorset.

Bovine gammaglobulin; Sigma Chemicals Co, Poole, Dorset.

PBS (pH 7.4) Dulbecco's, Oxoid Ltd, England.

Sodium Iodide ( $^{125}\text{I}$ ) Solution, Amersham, U.K.

Sodium Chromate ( $^{51}\text{Cr}$ ) Solution B.P., Amersham, U.K.

EDTA, Trisodium citrate, Glucose, Sephadex G15, Dextran Blue albumin, Chloramine T, KI, Sodium thiosulphate, Trichloroacetic acid were all purchased from Sigma Chemicals Co, Poole, Dorset.

Polydimethylsiloxane (PDS) as medical grade Silastic sheeting, non-reinforced; Dow Corning Co., Michigan, U.S.A.

Low density Polyethylene (PEE), Goodfellow Advanced Materials, Cambridge.

PHB, Mw 43,000; Marlborough Biopolymers, Teeside.

P(HB-HV), Mw 760K, 12.6% mole% HV, Marlborough Biopolymers, Teeside.

## Methods.

### 2.3.2. Polymer film preparation

A standardised preparation of polymer film (appendix 1) was used to ensure the reproducibility of the surface characteristics of the film. Irregular roughness and pitting, which could enlarge the true surface area, was minimal and the films were stored in clean vials to protect the surfaces from particulate contaminants. Polymer films were cast using a solvent evaporation process onto clean glass plates, with the result that the glass plate side of the film appeared smooth whereas the air dried side had a higher surface area and appeared rough. Prior to use films were cut into 3cm x 1cm strips and cleaned in an ultrasonic bath with three changes of double distilled water. Films were then pretreated with PBS buffer (pH 7.4) for three hours preceeding the adsorption study. It was important to ensure that there were no air bubbles at the surface of the film as an air/water interface would be expected to affect protein adsorption.

### 2.3.3. Buffer preparation

The adsorption of proteins is sensitive to pH, and to enable comparison of the adsorption measurements physiological ionic strength and pH was necessary. PBS (pH 7.4; I = 0.15M) was therefore used for all experimental work. This buffer was made up immediately before each experiment, and therefore remained free of biological contaminants which could degrade proteins and produce false adsorption data. The PBS was degassed by bubbling with helium to avoid air bubble formation at the surface of the films.

### 2.3.4. Protein preparation

Proteins were used as received with a purity of greater than 97% according to Sigma literature. Unlabeled protein solutions were made freshly prior to each experiment.

#### 2.3.5. Preparation of radiolabelled protein

The most popular method for  $^{125}\text{I}$  radiolabeling of proteins is the Chloramine T method where  $^{125}\text{I}$  is covalently bound to the tyrosine and to a lesser degree, the histidine residues of the protein molecule (Dube *et al.*, 1964 and Freendtender, 1971). The protocol used in this study was an adaptation of that originally described by Greenwood, Hunter and Glover (1963).

The entire radioiodination reaction was carried out at approximately 4°C using an ice bath. 10ul of Na  $^{125}\text{I}$  solution (equivalent radioactivity = 1mCi) was dispensed into a reaction tube followed by 10ul of 0.1N HCl to neutralise the solution. 200ul of PBS containing 10mg of the desired protein was added to the reaction tube followed by Chloramine T (10ug per mg of protein) and the reaction mixture allowed to stand for 10 minutes. After 10 minutes 20ul of  $\text{Na}_2\text{S}_2\text{O}_5$  (12.5mg/ml) and 20ul KI (25mg/ml) were added to the mixture to quench the reaction. The labeled protein  $^{125}\text{I}$  was separated from unbound  $^{125}\text{I}$  by gel filtration using a Sephadex G15 column (2.5cm diam. x 40cm).

#### 2.3.6. Separation of labeled protein from unreacted iodide.

Sephadex G-15 was hydrated with PBS pH 7.4 containing the appropriate protein and packed into the column. This procedure was adopted to saturate the column with unlabeled protein to minimize the adsorption of labeled protein during the separation procedure. The column was calibrated using a dextran blue albumin conjugate to establish the

elution time corresponding to the void volume. Subsequently the iodination mixture was transferred to the prepared column and eluted with PBS. 1ml fractions were collected in polypropylene tubes until both the protein and free  $^{125}\text{I}$  had been collected. The radioactivity in each tube was measured using a gamma counter.

#### 2.3.7a. Determination of free iodine.

The amount of free  $^{125}\text{I}$  in the labeled protein fraction was determined by precipitating one volume of the labeled protein with one volume of 50% trichloroacetic acid. The mixture was incubated at  $4^\circ\text{C}$  for 30 minutes then pelleted in an Eppendorf centrifuge at  $10,000 \times g$  for 3 minutes. 20ul of the supernatant was pipetted off and assayed using a gamma counter.

$$\% \text{ Free iodine} = \frac{\text{cpm in 20ul of supernatant}}{\text{cpm in 20ul of eluted samples}} \times 100$$

#### 2.3.7b. Calculation of the efficiency of iodination.

$$\% \text{ efficiency} = \frac{\text{dpm of 10ul of sample} \times \frac{\text{total volume collected}}{10\text{ul}}}{\text{dpm equivalent 1mCi}} \times 100$$

Calculation of total protein content of the iodinated samples was estimated using the Lowry assay.

#### 2.3.8. Rinse protocol

At the end of each experiment the film samples were removed from the reaction tubes and rinsed three times in 10ml PBS. The washings were assayed for  $^{125}\text{I}$ . Films were soaked overnight in PBS and the wash assayed to establish the required rinse protocol. Proteins are adsorbed onto solid surfaces in at least two forms, a) a loosely bound relatively rapidly exchanged fraction and b) a more tightly bound, slowly or nonexchanging fraction. It appeared that most loosely bound protein was removed by three washes with PBS and in most cases an overnight soak did not result in further desorption.

#### Experimental methods.

##### 2.3.9. Preferential adsorption.

Preferential adsorption is defined to distinguish between the difference in adsorption between labeled protein and its unlabeled counterpart. If labeled protein has a higher affinity than unlabeled a 'preferential' adsorption takes place. To ensure that radioiodination of the proteins did not affect their adsorption properties, a test for preferential adsorption was carried out. The adsorption of protein was determined using a wide range of specific activities by changing the ratio of the labeled to nonlabeled protein. If labeled protein was adsorbed to the same extent as non-labeled protein the net adsorption would be independent of the quantities of labeled protein used. However if  $^{125}\text{I}$  protein was preferentially adsorbed, the apparent adsorption would be expected to increase as the ratio increased. Experiments were carried out for a series of solutions with constant net protein concentration (2mg/ml) which varied in the fraction of the total which was radiolabeled (0.001-0.1). After 2 hrs incubation at 37°C, the films were rinsed and the polymer film using a gammacounter. Results were expressed as

the amount of labeled protein adsorbed to polymer film in ug/cm<sup>2</sup>.

#### 2.3.10. Adsorption isotherms

Adsorption isotherm were determined by exposing the polymer films to a series of concentrations (0.001 to 2mg/ml) of each protein for 2 hours.

#### 2.3.11. Competitive adsorption

Competitive adsorption to the polymer films was investigated using a series of binary protein solutions in which the concentration of one protein (non-labeled) was varied and the concentration of a second (labeled) protein was kept constant. The ratios of unlabeled protein (I) to labeled protein (II) were 0, 0.001, 0.01, 1.0, 10, 20, 70 and 100. After 2 hrs equilibration the films were thoroughly rinsed as described in section 2.3.8. and counted using the gamma counter.

#### 2.3.12. Preparation of platelet suspension and isotope labeling.

40ml of rabbit blood was collected by gravity through a 19G needle into a polypropylene tube containing 1 part tri-sodium citrate solution (3.8%) to 8 parts of blood. Platelet rich plasma (PRP) was collected from the supernatant of blood which was centrifuged at 1050rpm for 20 minutes. The platelet density per ml was determined using a thrombocytometer and adjusted to a final concentration of  $4 \times 10^5$ /ml with PBS. 10ul of <sup>51</sup>Cr Sodium Chromate solution of specific activity 1mCi was added to the PRP and incubated for 1 hr at 37°C. To stop the reaction 3ml EDTA (1mM) and glucose (5mM) was added and the prp was then rinsed three times with PBS to remove unbound <sup>51</sup>Cr. The <sup>51</sup>Cr resides in the plasma membrane and cytoplasm, (Steiner and Baldini, 1970) and was assayed using a gamma counter.

### 2.3.13. Platelet adhesion experiments.

Polymer films were exposed to the labeled PRP for time periods of 1 minute to 60 minutes to establish the kinetic profile of platelet adhesion for each surface. The adhesion number was defined : Adhesion number = number of platelets per  $10^4 \text{ } \mu\text{m}^2$ . The adherence of platelets to the polymer surfaces was analysed according to Whicher and Brash (1978) and determined by comparing the radioactivity of the surface with that of an aliquot of suspension of known platelet count. The surface count of  $^{51}\text{Cr}$  can be used to compute the number of platelets adhering per unit area when compared to the suspension count.

$$\text{Platelet adhesion no. per unit area} = \frac{\text{no. of platelets per ul} \times {}^{51}\text{Cr film surface count} \times 10^4}{{}^{51}\text{Cr count per ul PRP} \times \text{polymer film area (}\mu\text{m}^2\text{)}}$$

After establishing the kinetic profile the polymer films were precoated with either albumin, fibrinogen or gammaglobulin for three hrs and the platelet adhesion number determined as described.

## Assessment of soft tissue inflammatory response : *in vivo*. Materials and Methods

### 2.4.1. Materials

Sterile 1ml syringes; Plastipak, Beckton and Dickinson, Leicester.

Sterile 25 gauge needles; Plastipak, Beckton and Dickinson, Leicester.

PHB (Mw 43,000); Marlborough Biopolymers, Teeside.

P(HB-HV) (Mw 760,000) 12.6 mole% HV; Marlborough Biopolymers, Teeside.

Hydroxybutyrate and hydroxyvalerate as sodium salts; Sigma Chemical Co., Poole, Dorset.

Methylcellulose; Aldrich Chemicals Ltd, Gillingham, Dorset.

Polyacrylic acid; Aldrich Chemicals Ltd, Gillingham, Dorset.

Polymethylmethacrylate; Aldrich Chemicals Ltd, Gillingham, Dorset.

Formalin; BDH Chemicals Ltd, Poole, Dorset.

Zymosan; Sigma Chemical Co, Poole, Dorset.

Evan's Blue Injection Ampoules (25mg/5ml); Harvey Laboratories Inc. Philadelphia, PA.

Histamine, 5HT; Sigma Chemicals Co, Poole, Dorset.

Concentrated Hydrochloric acid and sodium hydroxide; Fison's Laboratories, Loughborough.

Alkaline and acid phosphatase kits (Procedure no. 104); Sigma Chemicals Co, Poole, Dorset.

Preparation of polymer films and microspheres is described in appendix 1.



## 2.4.2.Methods

### 2.4.2.1. Preparation of injections.

All materials to be injected were sterilised by autoclaving at 121°C for 15 minutes, ensuring that the solutions or suspensions were not adulterated by the sterilisation process. Using methods documented in the British Pharmacopeia, Volume II, 1988, 10% of each injection was tested for sterility and pyrogenicity (see appendices 3 and 4 respectively). Products which gave positive results, i.e. microbial growth or gelation of the Limulus amoebocyte lysate, were rejected as false information would be derived from such products.

### 2.4.2.2. Histological studies.

#### Injection procedure.

0.3ml of each test material was injected intramuscularly into the gastrocnemius muscle of male Wistar rats (200g, 10 weeks old), using a single injection per rat. After 7 or 14 days the animals were killed by dislocation of the neck, the injection sites located and a gross macroscopic evaluation carried out by visual inspection. The tissue at the injection sites was carefully excised and then fixed in 10% phosphate buffered formalin prior to preparation for histological evaluation.

#### Histological preparation.

The fixed muscle tissues were embedded in epoxy resin, sectioned, mounted then stained with haematoxylin and eosin. Each tissue section was examined under high and low power using a Weiss microscope. This procedure was kindly carried out by Dr McCleod at the Royal United Hospital.

#### 2.4.2.3. Assessment of increased vascular permeability.

Male Wistar rats, 100-150g in weight, were lightly anaesthetised with ether and their tails warmed in water at 45-50°C to dilate the tail vessel. Intravenous injections (0.4ml) of Evan's Blue (25mg/5ml) were introduced into a lateral tail vein. Intradermal injections (0.2ml) of the polymer suspensions (20mg polymer/ml) and monomer solutions (10 and 100mg/ml) in PBS were introduced into the abdominal skin previously shaved with commercial animal clippers. The conscious animals were left for 2, 3 or 4 hours at room temperature before killing by stretching the neck. After killing, the area of dye leakage was excised and extracted over 24 hours with a mixture of 7ml acetone and 3ml 0.5% sodium sulphate solution. After this period the tissue was removed and, following centrifugation to remove debris, the concentration of dye in the supernatant was determined by UV absorbance at 620nm using a Perkin-Elmer 550S spectrophotometer. To allow for variations in background "blueing" of the rats, a reference blank was obtained from each rat by excision and extraction of a piece of abdominal skin taken from outside the locality of the injection. The absorbances measured were converted to concentration with the use of Beer-Lambert calibration curve. Known irritants, 5HT (1mM), histamine (1mM), 1M HCl and 0.1M NaOH were also injected intradermally and the resulting degree of increased vascular permeability assessed as above for use as comparative indices.

#### 2.2.2.4. Assay of cellular activities.

##### Animal treatment

A 10mg/ml injection of PHB microspheres (approximate diameter 40µm) was prepared using a 1% methylcellulose and 0.9% saline injection vehicle. The injection mixture was sterilised in an autoclave at

121°C for 15 minutes. On cooling the injection mixture was sonicated to redisperse the microspheres and prevent further aggregation. A control injection mixture of vehicle only was also prepared as above.

Male Wistar rats, 150-200g in weight, were fed on Labure pellets with water ad libitum. Into the right thigh muscle of each rat 0.3ml of the polymer suspension was injected using a 25 gauge needle. Other rats were similarly treated with 10mg/ml suspensions of P(HB-HV) and 10 or 100mg/ml solutions of either monomer (HB or HV) as its sodium salt. The left thigh of each animal was used as the control by injection of the vehicle alone.

At intervals post injection (1,2,4,6,8,10 or 40 days) the rats were anaesthetised with ether, cardiac bled then immediately killed. 5ml of blood from each rat was collected, centrifuged for 10 minutes at 3000rpm and stored on cracked ice ready to be assayed. The right and left thigh muscles were immediately removed, separately weighed and stored in 0.9% saline solution on cracked ice. Each muscle was homogenised thoroughly, the homogenate centrifuged for 10 minutes at 3000rpm and the clear tissue extract removed for assay. The procedure was repeated with known inflammatory materials; zymosan (100mg/ml), polyacrylic acid (PAA) (100mg/ml), and a known biocompatible polymer polymethylmethacrylate (PMMA) (100mg/ml), which is used extensively for the manufacture of contact lens. These positive and negative control injections were presented to the tissue as solutions (PAA) or suspensions of the powder (zymosan and PMMA). Films of PHB and P(HB-HV) (2cm x 1cm) were also used in this study to evaluate the effect of the physical form of the implant on the intensity and duration of the inflammatory response. The films were sterilised by autoclaving and implanted into the muscle tissue using sterile blunt forceps to create a pocket between the muscle fibres. The wound was

sealed with sterile animal clips. PMMA films were used as the control in this experiment.

#### Enzyme assay

The extracellular enzyme activities of exudate samples were determined using a colorimetric method. Both assays involved enzyme reaction with p-nitrophenylphosphate as the substrate. A 2-amino-2-methyl-1-propanol buffer at pH 10.3 was used for assay of alkaline activity and a citrate buffer at pH 4.8 was appropriate for the acid phosphatase activities (figure 3.1). The absorbance of the degradation product p-nitrophenol, was measured at 398nm using a Perkin-Elmer 550S spectrophotometer.

CHAPTER 3. The preliminary assessment of the biocompatibility of PHB, P(HB-HV) and their monomers using cell culture.

3.1 Introduction

Many *in vitro* systems have become available for use as primary toxicological screens for new biomaterials in relation to their potential use in medicine and dentistry. The history of the application of *in vitro* screening programmes has been the subject of a number of comprehensive reviews (Pomerat, 1954; Schindler, 1969; Tardiff, 1978; Rees, 1980 and Ekwall, 1980). Cell culture assays are currently the most popular *in vitro* tests for acute toxicity evaluation on the basis that an observed *in vitro* event may also occur *in vivo*. Cultured mammalian cells offer a number of advantages over *in vivo* systems for the assessment of the biomaterial toxicity. These include sensitivity and rapidity of response, reproducibility of observations (as the cells are from a phenotypically and genetically uniform stock maintained in a precisely defined and highly controllable environment), ease of experimentation and also relative inexpense in comparison to animal experiments, (Grisham and Smith, 1984).

The experimental protocol used in cell culture toxicity testing depends on the choice of cell line. Important considerations when choosing the cell line include the time span of the project. A project that is to be run over several months requires the use of a finite cell line, and since different cells have differing abilities to multiply, some element of choice is given. Human cells in culture for biomaterial toxicity testing have been used by a number of investigators (Rae, 1986), however due to the limited life span of these cells it was impossible to complete a set of experiments on

cells from a single source. It is also important to decide whether the cells will be grown in monolayers (and, if so, to ensure that the cell line selected does not show a tendency to overgrow), or whether the cells will be grown in suspension. Cells which are derived from dissociated tumours will grow and divide when maintained in suspension, but cultures of this type have had a limited application in biocompatibility testing. With the above considerations in mind an established cell line was chosen for this work. CHO-K1, isolated from Chinese Hamster Ovary (Puck *et al.*, 1958), have been used by Levis and Majone (1979) in toxicity testing and is the cell line of choice in this study.

Most toxicity testing is performed on a soluble extract of the material under investigation, (Grasso *et al.*, 1973; British Standards 5736, 1981 and United States Pharmacopeia, 1975) or on the material itself with its components coming into direct contact with the cells in monolayer culture. Homsey (1970) exposed a number of polymers in a pseudoextracellular fluid for 62hr at 115°C and then used aliquot portions of this to prepare the nutrient medium for newborn mouse heart tissue. Using this method it was shown that certain polymers such as polyacrylic acid exhibited a moderate degree of cytotoxicity. Some investigators have used the biomaterial itself as the substratum for cell attachment, (Campbell *et al.*, 1941). However the growth of cells directly on a surface will not be a suitable technique for the investigation of all biomaterials as in some cases e.g. materials used in blood pumps and indwelling catheters, it is not desirable for the cells to become attached to the surface. Other investigators, Rosenbluth *et al.*, (1965) and Lydon *et al.* (1985) allowed polymers to come into direct contact with cell monolayers. However, polymeric materials which have a density less

than that of the tissue culture medium tend to float and do not come into direct contact with the cells. This problem can be overcome by growing cells on a solid substratum under growth medium which contains a gelling agent. The test material is placed on top of the solid gel and the cytotoxic effects measured as the components of the material diffuse through the solid media to the monolayer of cells beneath, this is known as the agar overlay technique first used by Guess *et al.* (1965) and since by Grasso *et al.*, 1973, Peling *et al.*, 1973 and Chawla *et al.*, 1982. However there are problems with the agar overlay technique, since the agar may impede diffusion of the soluble products and/or moieties of the test material thereby decreasing the sensitivity of the assay. Wennberg *et al.*, (1979) attempted to overcome this problem by growing cells as a monolayer on one side of a membrane filter placed on top of an agar overlay and then the test biomaterial was placed on the other side of the filter. This technique altered the growth of the cells in monolayer due to the pores in the filter. Because of these potential problems it was decided to use in this study a direct contact method to study the cytotoxicity of PHB, P(HB-HV) and their degradation products.

End points that can be quantified as cellular manifestations of toxicity vary in degrees of precision and sensitivity. The appropriate technique is normally governed by choice of cell line. A major class of methods involve the measurement of cellular integrity, either by dye exclusion, (Phillips, 1973) or by the retention of radioactive chromium, (Ronai, 1969). However, permeability of the cell membrane to such markers does not unambiguously demonstrate cell death, as some cells are able to repair the underlying damage to the cell membrane or ion pumps and re establish volume control (Castellot *et al.*, 1978; Kucera and Paulus, 1982). Changes in the cellular

morphology and histology have also been used, (Grasso, 1973) however, such methods can only be used to obtain a qualitative end point since the interpretation of results is often subjective. Biochemical assays e.g. the release of lysosomal enzymes such as lactate dehydrogenase (LDH) and the measurement of cellular respiration have also been used to quantify manifestations of cellular toxicity (Rae, 1975 and 1981). However, a major disadvantage of biochemical testing for LDH activity is that calf serum contains appreciable amounts of LDH which will vary for different batches of sera used in multiple experiments. A more accurate method of determining cell death/toxicity is that of scoring the cell population either by direct cell count using a haemocytometer or indirectly by assay of cellular protein or DNA. Direct cell counts were used by Pappas and Cohen (1968) and Mital and Cohen (1968) when studying the toxicity of metal particles in tissue culture. A direct cell count method was one of the methods used in this present study to determine the cellular toxicity of PHB, P(HB-HV) and their degradation products.

The following chapter describes the estimation of cellular toxicity caused by the polymers and their degradation products in direct contact with a monolayer of CHO-K1 cells using:

- 1) a direct cell count method with construction of growth curves,
- 2) a replica plating method using a colony formation assay.



### 3.2 Results

3.2.1. The effect of PHB, P(HB-HV) and their monomers on the growth parameters of CHO-K1 cells.

For each of the treated cultures the lag and population doubling time ( $t_{1/2}$ ) was calculated to enable a direct comparison between control and treated cultures. The lag time was calculated by extrapolation of the exponential portion of the curve back to the axis of the original seeding density. The population doubling time determined from the exponential part of the curve was calculated from the following equation:

$$\text{Log } N_t = \text{Log } N_0 + kt \text{ Log } 2$$

where  $N_t$  is the number of cells at time  $t$ ,  $N_0$  is the number of cells at time 0,  $t$  is the time in hours,  $k$  is the exponential growth rate constant and  $1/k$  gives the population doubling time. The effect on the growth parameters of CHO-K1 cells grown in the physical presence of PHB, P(HB-HV), PMMA and PAA over a 60 hr period is shown in figure 3.1. From these growth curves the lag time and population doubling time were calculated (table 3.1). Statistical analysis between groups was performed using Student's 't' test in all cases.

The growth parameters indicated that the PHB treated cultures did not differ ( $p > 0.05$ ) from the control culture, indicating good cell biocompatibility. The lag and population doubling times of the CHO-K1 cells grown in the presence of P(HB-HV) polymer films were greater when compared to the control growth parameters, but were not significantly different. PMMA treated cultures showed a significant difference ( $p < 0.02$ ) in the lag time of the PMMA treated cultures, however no significant difference in  $t_{1/2}$  was found between treated cultures and control cultures. As expected the PAA gel was found to

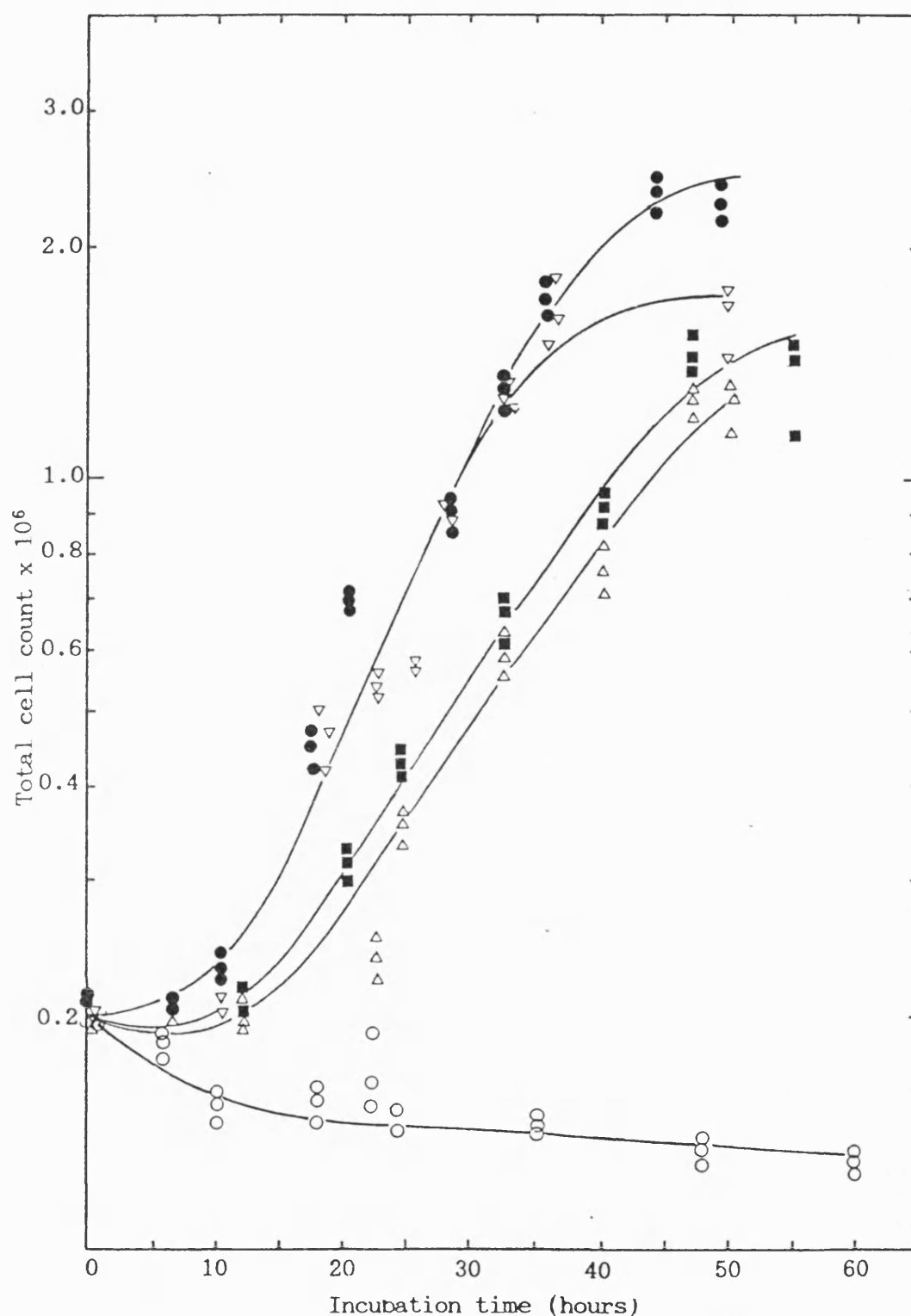


Figure 3.1. Growth curves of CHO-K1 cells grown in Hams F10 + 5% FCS in the presence of polymer films (4%w/v), PHB ( $\nabla$ ), PAA ( $\circ$ ), P(HB-HV) ( $\blacksquare$ ) or PMMA ( $\triangle$ ). The growth curve of cells grown in the absence of any polymer is also shown ( $\bullet$ ). Each point represents the mean of 3 experiments. The s.d. in each case was  $< 5\%$  of the mean.

Treatment	Lag time (hr)	Mean (s.d.) n = 3	T <sub>1/2</sub> (hr)	Mean (s.d.) n = 3	r
Control	7.34	8.33 (2.21)	9.93	10.48 (1.40)	0.96
	11.39		9.10		0.96
	6.25		12.40		0.96
PHB	8.25	8.14 (1.15)	9.23	9.82 (0.53)	0.96
	6.68		9.71		0.96
	9.50		10.52		0.96
P(HB-HV)	12.22	11.93 (0.55)	12.45	11.70 (0.55)	0.99
	12.41		11.15		0.99
	11.16		11.50		0.99
PMMA	20.94	17.77 (3.58)	7.64	9.19 (2.34)	0.99
	12.77		12.49		0.99
	19.60		7.43		0.99
PAA	cell death		cell death		----

Table 3.1. The effect of polymer films (4%w/v) on the growth characteristics of CHO-K1 cells grown in Ham's F10 and 5% FCS.

T<sub>1/2</sub> = Population doubling time.

r = Correlation coefficient.

inhibit cell growth and a degree of cytotoxicity was observed after only 10hr culture. Following a 48 hr pre-treatment with each of the polymer films the effect on the growth of CHO-K1 cells is shown in figure 4.2. and the calculated growth parameters given in table 4.2.

These results indicate good cell biocompatibility of both the homopolymer copolymer and PMMA following 48hr pre-treatment, as the growth parameters displayed no significant difference ( $p > 0.05$ ) between the test polymers and the control culture. This suggests that the effect of the physical presence of copolymer and PMMA films on the growth of CHO-K1 cells caused the increase in lag time when cells were cultured in their continual presence. Predictably, as PAA induced cell death after 10 hr of culture then after 48hr pre-treatment no cell growth occurred.

The effect of the monomers, HB and HV as their sodium salts on the growth of CHO-K1 cells is shown in figures 3.3 - 3.5 and the calculated growth parameters presented in tables 3.3 and 3.4. The effect of the monomers on the growth of CHO-K1 cells indicated that at concentrations above 10mg/ml (HB) and 15mg/ml (HV) moderate to severe cytotoxicity occurred as the lag time and  $t_{1/2}$  increased. To establish whether the pH or osmolarity of these monomers at certain concentrations had any effect on the cytotoxicity, sucrose and sodium chloride were used at concentrations that gave similar pH and osmolarity readings to the monomers i.e. cell compatible concentrations (<10mg/ml HB and <15mg/ml HV) and cytotoxic concentrations (>10mg/ml HB and >15mg/ml HV). Figure 3.6. shows the calibration curve for osmolarity used to determine the concentrations of sucrose and sodium chloride to be used. From this concentrations of sucrose and sodium chloride that gave similar osmolarity readings to the cell compatible concentrations of the

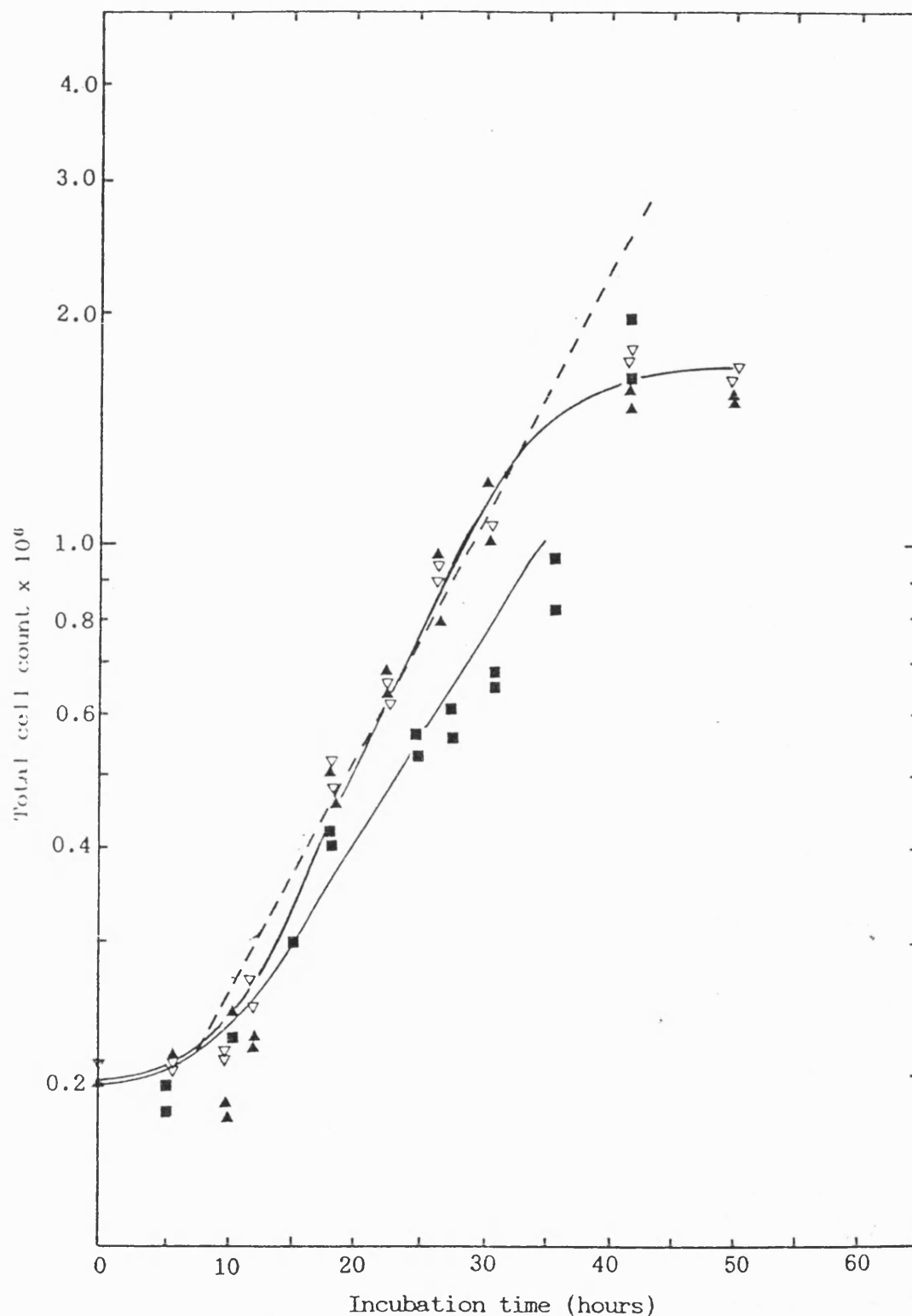


Figure 3.2. Growth curves of CHO-K1 cells grown in Hams F10 + 5% FCS following a 48 hour exposure to polymer films (4%w/v), PHB (▽), P(HB-HV) (▲), or PMMA (■). A regression line for control cells (-----) is included for comparative purposes. Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.

Treatment	Lag time (hr)	Mean (s.d.) n = 3	T <sub>1/2</sub> (hr)	Mean (s.d.) n = 3	r
Control	7.34		9.93		0.96
	11.39	8.33 (2.21)	9.10	10.48 (1.40)	0.96
	6.25		12.40		0.96
PHB	6.74		11.16		0.99
	6.65	6.80 (0.15)	8.65	9.38 (1.27)	0.99
	7.00		8.32		0.98
P(HB-HV)	8.06		11.26		0.99
	6.25	7.79 (1.16)	11.44	11.32 (0.08)	0.98
	9.05		11.26		0.98
PMMA	6.50		7.64		0.99
	7.50	7.50 (1.00)	12.49	9.19 (2.34)	0.99
	6.50		7.43		0.99
PAA	no growth/ cell death		cell death		cell death

Table 3.2. The effect of 48 hrs pre-treatment with polymer films (4%w/v) on the growth characteristics of CHO-K1 cells grown in Ham's F10 and 5% FCS.

T<sup>1/2</sup> = Population doubling time.

r = Correlation coefficient.

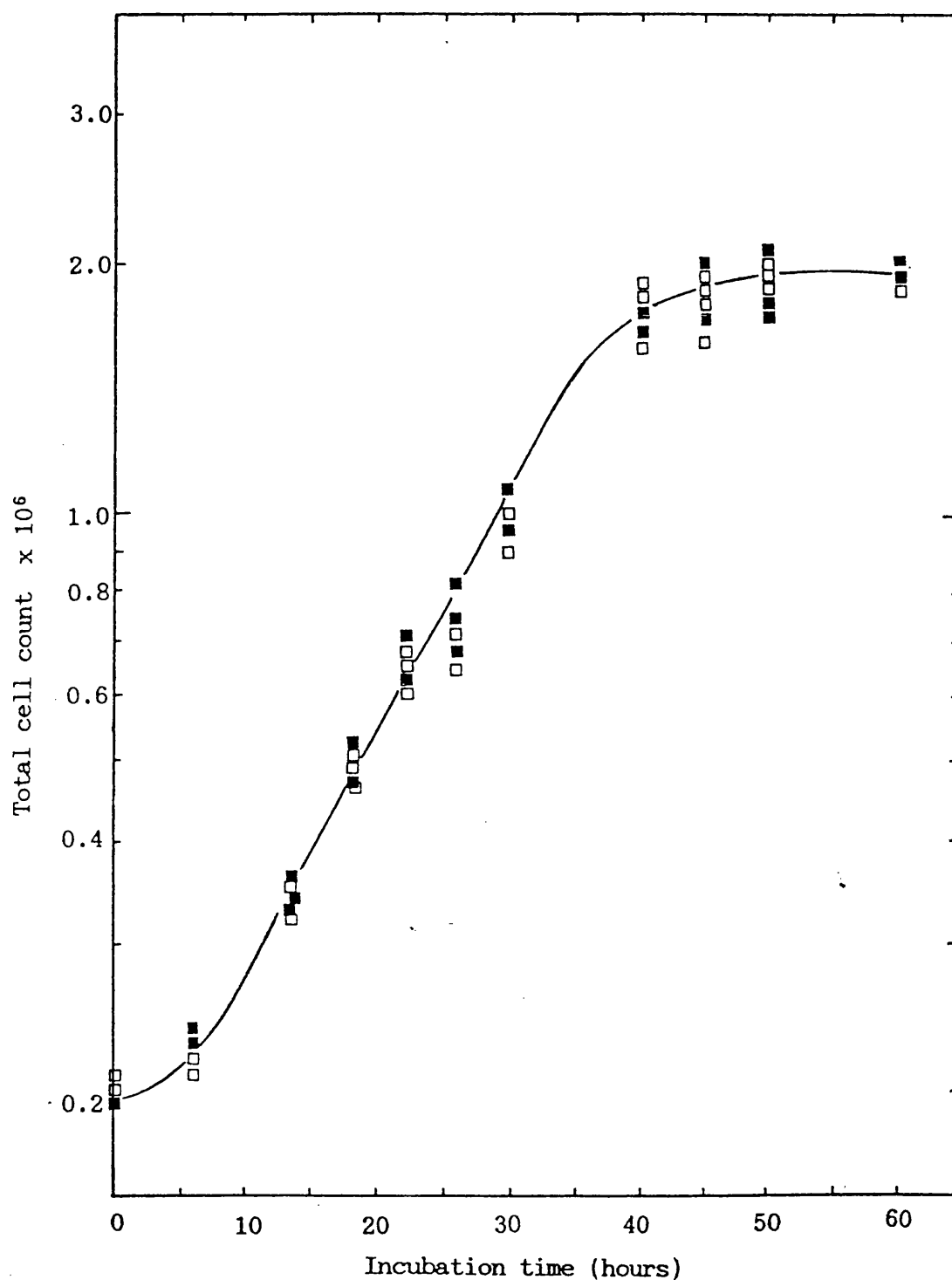


Figure 3.3. Growth curves of CHO-K1 cells grown in Hams F10 + 5% FCS in the presence of hydroxybutyric acid. Cells were grown with 3 mg/ml or 5 mg/ml hydroxybutyric acid (■, □, respectively). Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.

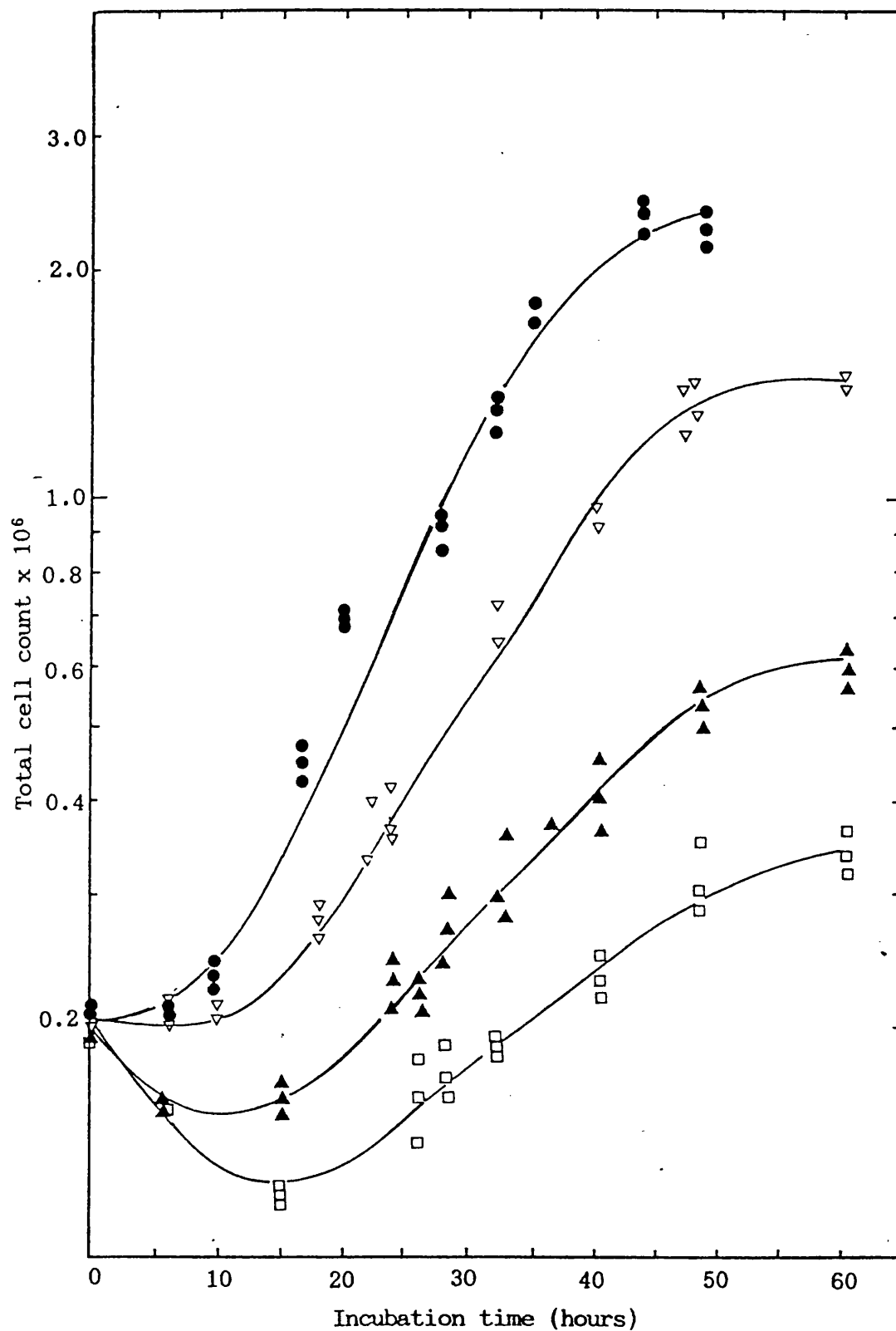


Figure 3.4. Growth curves of CHO-K1 cells grown in Hams F10 + 5% FCS in the presence of 10, 15 or 20 mg/ml hydroxybutyric acid ( ▽ , ▲ , □ ). The growth curve of cells grown in the absence of hydroxybutyric acid is also shown ( ● ). Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.



Treatment	Lag time (hr)	Mean (s.d) n = 3	T <sub>1/2</sub> (hr)	Mean (s.d) n = 3	r
Control	7.34	8.33 (2.21)	9.93	10.48 (1.40)	0.96
	11.34		9.10		0.96
	6.25		12.40		0.96
HB 1mg/ml	9.10	9.90 (0.61)	8.23	7.59 (0.47)	0.96
	10.02		7.43		0.96
	10.57		7.11		0.96
3mg/ml	12.60	12.18 (0.36)	7.60	7.41 (0.19)	0.98
	12.20		7.49		0.97
	11.73		7.15		0.98
5mg/ml	13.05	12.05 (0.71)	7.53	7.60 (0.10)	0.98
	11.55		7.63		0.99
	11.55		7.63		0.99
10mg/ml	15.28	14.08 (1.00)	10.75	10.92 (0.30)	0.98
	14.14		11.30		0.98
	12.82		10.70		0.99
15mg/ml	22.96	20.23 (2.10)	14.33	17.07 (2.10)	0.97
	19.84		17.37		0.98
	17.90		19.52		0.96
20mg/ml	36.47	34.04 (2.40)	20.46	22.88 (4.70)	0.99
	31.60		18.73		0.99
	30.40		29.45		0.98
25mg/ml	cell death		cell death		----

Table 3.3. The effect of 3 -Hydroxybutyrate (as sodium salt) on the growth characteristics of CHO-K1 cells grown in Ham's F10 and 5% FCS.  
T<sub>1/2</sub> = Population doubling time.

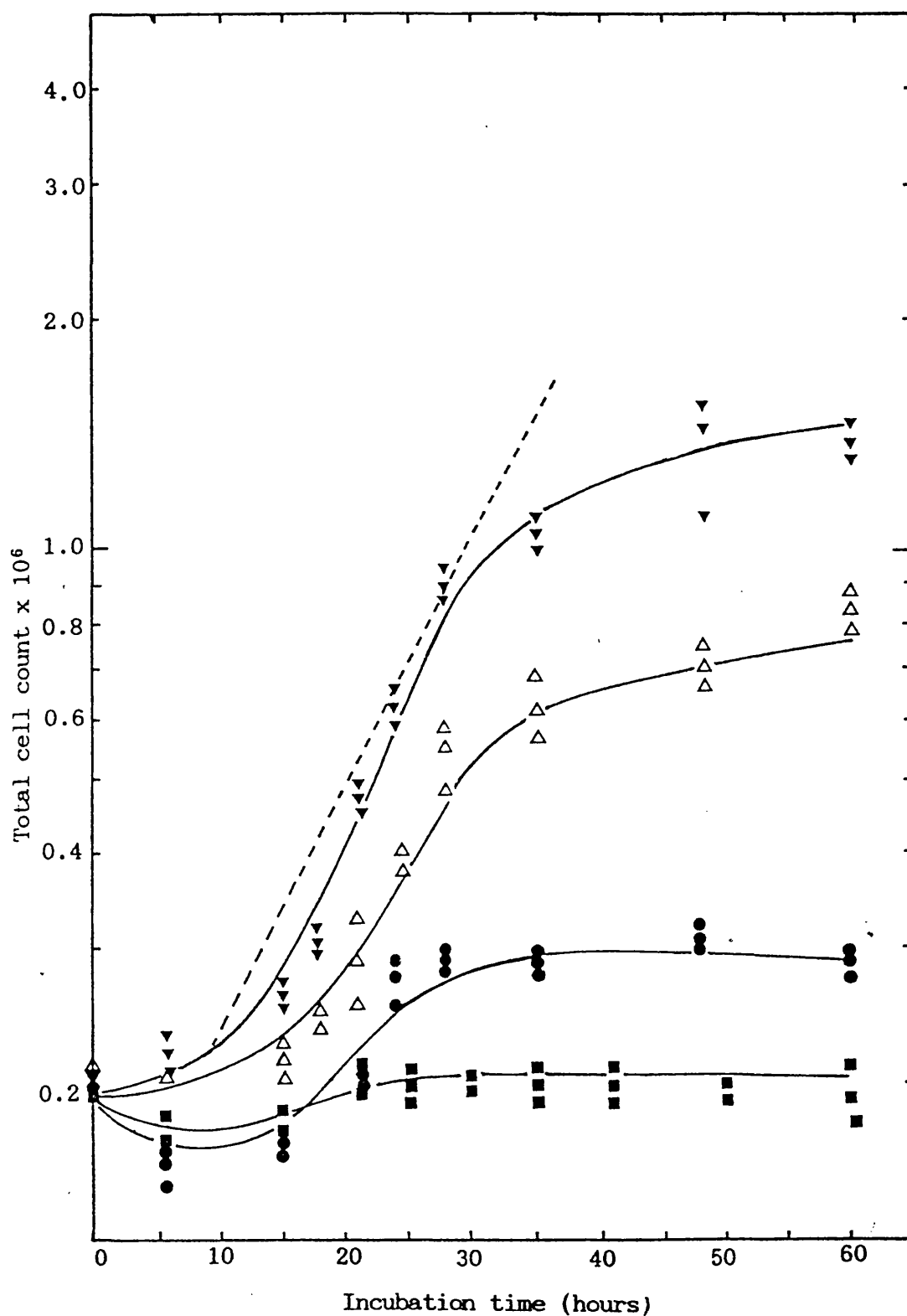


Figure 3.5. Growth curve of CHO-K1 cells grown in Hams F10 + 5% FCS in the presence of 10, 15, 20, or 20 mg/ml hydroxyvaleric acid (▼, Δ, ●, ■). A regression line for control cells (-----) is included for comparative purposes. Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.

Treatment	Lag time (hr)	Mean (s.d.) n = 3	T <sub>1/2</sub> (hr)	Mean (s.d.) n= 3	r
Control	7.34	8.33 (2.20)	9.93	10.48 (1.40)	0.96
	6.25		9.10		0.96
	11.39		12.40		0.96
HV 1mg/ml	5.50	7.60 (1.50)	10.75	9.70 (0.60)	0.96
	8.60		9.41		0.97
	8.60		9.41		0.95
3mg/ml	8.60	8.60 (0.00)	9.40	9.40 (0.00)	0.96
	8.60		9.40		0.96
	8.60		9.40		0.96
5mg/ml	8.30	8.50 (0.14)	9.70	9.50 (0.14)	0.96
	8.60		9.40		0.96
	8.60		9.40		0.96
10mg/ml	9.60	9.60 (0.30)	6.50	6.65 (0.05)	0.98
	10.00		6.70		0.98
	9.30		6.70		0.98
15mg/ml	15.70	15.10 (1.30)	9.70	9.10 (0.80)	0.96
	16.20		7.90		0.96
	13.30		9.50		0.96
20mg/ml	cell death		cell death		----
25mg/ml	cell death		cell death		----

Table 3.4. The effect of 3 -Hydroxyvalerate (as the sodium salt on the growth characteristics of CHO-K1 cells grown in Ham's F10 and 5% FCS.

T<sub>1/2</sub> = Population doubling time.

r = Correlation coefficient.

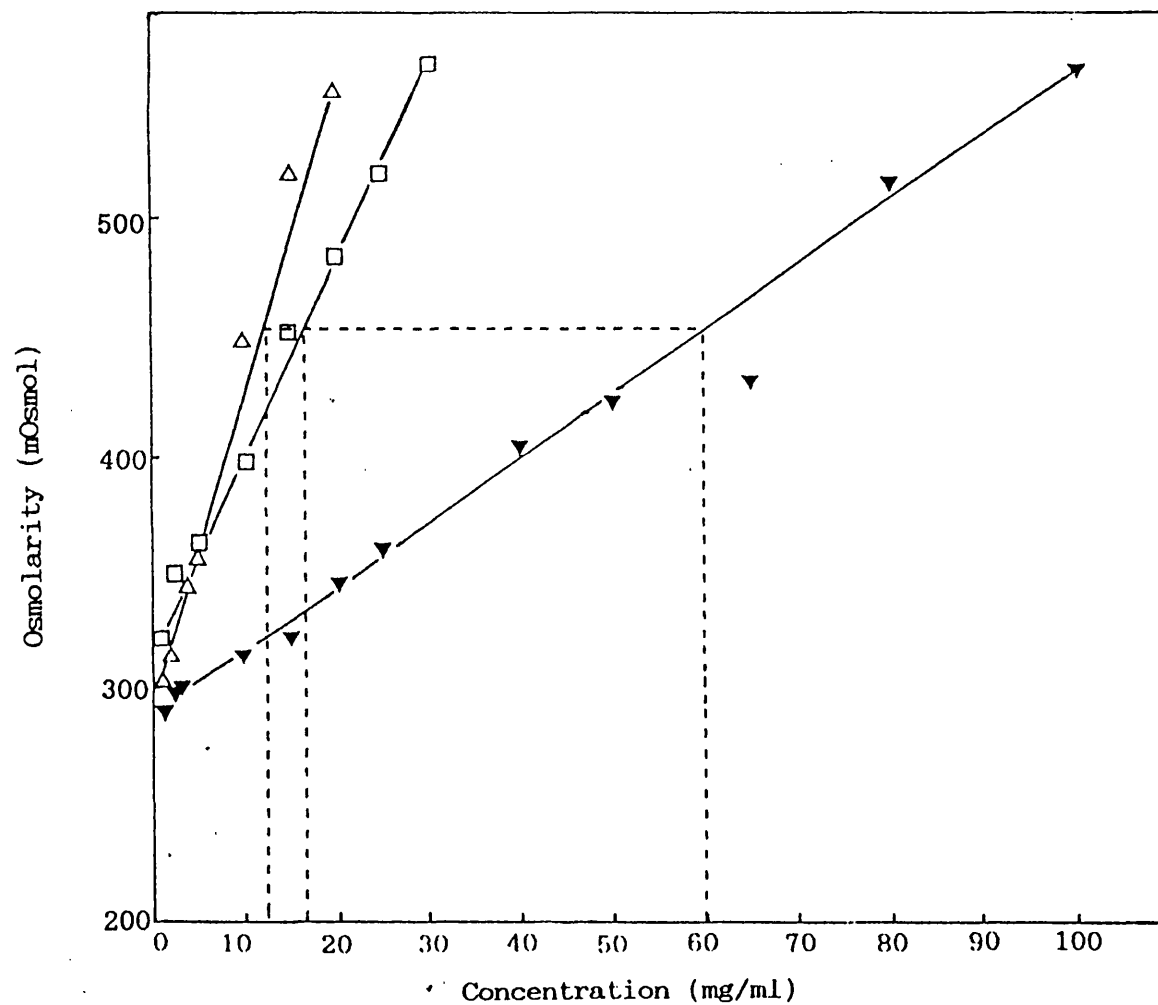


Figure 3.6. Iso-osmolarity estimation of sucrose (▼), hydroxybutyric acid (Δ) and hydroxyvaleric acid (□) solutions. Determinations were carried out using a calibrated Camlab freezing point osmometer ( ref. section 2.1.16.). Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.

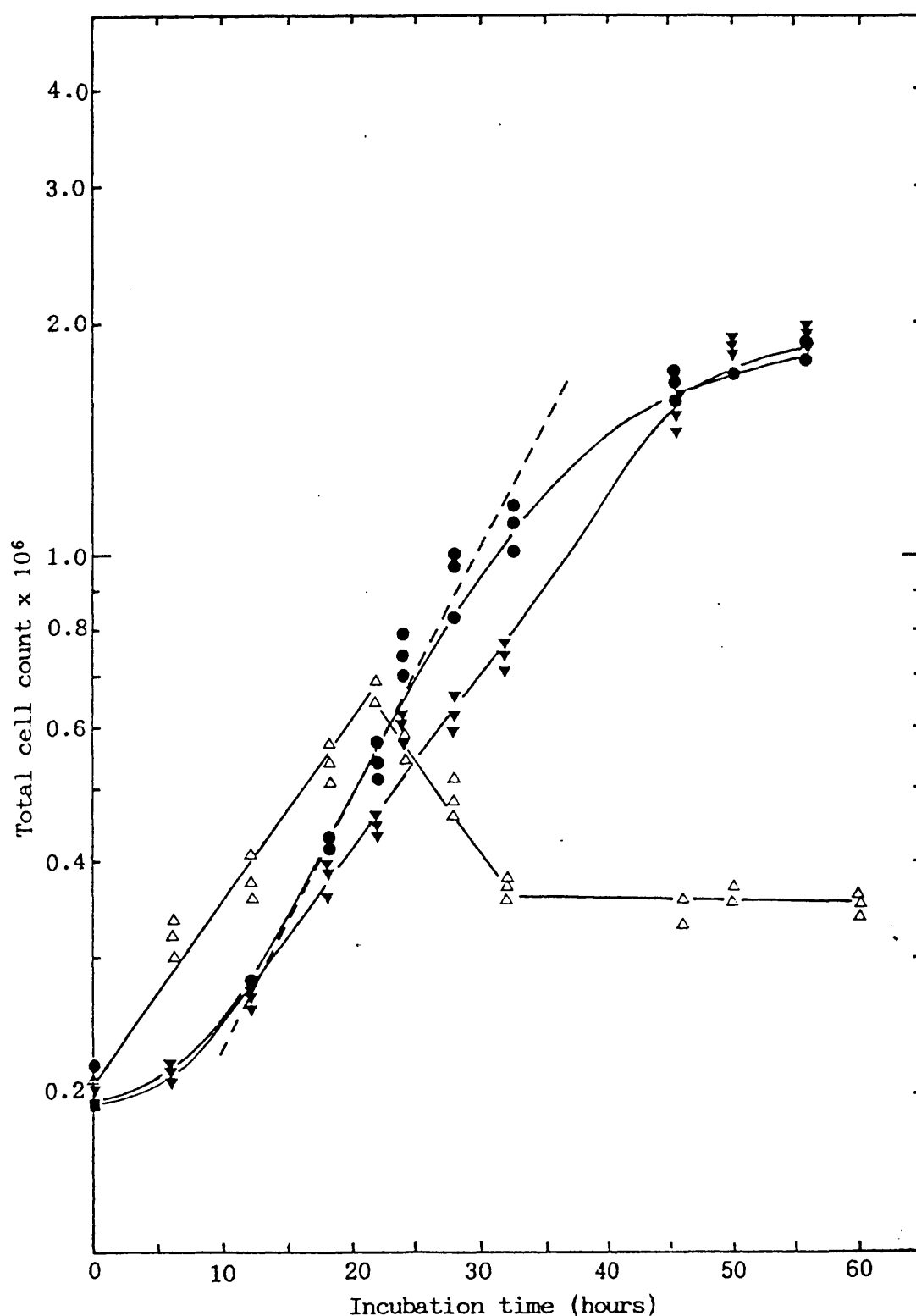


Figure 3.7. Growth curves of CHO-K1 cells grown in Hams F10 + 5% FCS in the presence of 12.5 mg/ml NaCl (●) and 60 mg/ml NaCl (●) and 60 mg/ml or 100 mg/ml sucrose (▼, △). A regression line for control cells (-----) is included for comparative purposes. Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.

Treatment	Lag time (hr)	Mean (s.d) n = 3	T <sub>1/2</sub> (hr)	Mean (s.d) n = 3	r
Control	7.34	8.33 (2.20)	9.93	10.48 (1.40)	0.96
	11.39		9.10		0.96
	6.25		12.40		0.96
Sucrose 60mg/ml	5.85	4.11 (1.20)	14.28	14.76 (0.40)	0.90
	3.36		15.16		0.90
	3.12		14.85		0.90
100mg/ml	cell death		cell death		----
NaCl 12.5mg/ml	8.70	7.67 (0.70)	10.03	10.00 (0.10)	0.99
	7.20		10.11		0.96
	7.10		9.87		0.96
25mg/ml	cell death		cell death		----

Table 3.5. The effect of NaCl and sucrose of equivalent osmolarities to 10 and 15mg/ml of HB and HV on the growth characteristics of CHO-K1 cells grown in Ham's F10 and 5% FCS.

T<sub>1/2</sub> = Population doubling time.

r = Correlation coefficient.

monomers are 60mg/ml sucrose and 12.5mg/ml NaCl (= 10mg/ml HB and 15mg/ml HV) and 100mg/ml sucrose and 25mg/ml NaCl (= 15mg/ml HB and 20mg/ml HV) respectively. The growth curves were constructed as before (figure 3.7) and the growth parameters calculated (table 3.5).

Sucrose and NaCl at concentrations of equivalent osmolarity to the cytotoxic concentrations of HB and HV were seen to exhibit cytotoxic effects on CHO-K1 cells. The treatment of the cells with equivalent osmolarity to concentrations which were cell compatible, had similar calculated population doubling times, however there was a significantly shorter lag time ( $p < 0.05$ ) required for the cells grown in 60mg/ml sucrose. This could be due to the surplus sugar and energy provided by a culture medium rich in sucrose, which would have enabled the cells to recover from trypsinization and allow attachment to the culture flasks more rapidly. These results indicate that osmolarity does affect the growth of CHO-K1 cells in monolayer culture.

### 3.2.2. Replica Plating

Transfer efficiencies from plate to polyester cloth:

CHO-K1 cell lines were seen to have a basal colony-forming efficiency of 60-70%. Three or four high resolution copies could be generated from a single master plate, with 100% transfer efficiencies from the plastic culture dishes to the overlays. At this stage the CHO colonies were 2-4mm in diameter.

Effect of homopolymer, copolymer and monomers on the replica plates of colonies of CHO-K1 cells.

Table 3.6. shows the effect of the polymers and their monomers on the replicate CHO colonies over 72 hrs. After 72 hours contact with the

Table 3.6 The percentage survival of CHO-K1 colonies following exposure to either the polymers or monomers.

% survival =  $\frac{\text{no of colonies on treated replica plates}}{\text{no of colonies on untreated master replica plate (control)}}$

Treatment	Percentage survival (S.D.) n=3		
	24 hr exposure	48 hr exposure	72 hr exposure
PHB film	98.9 (0.46)	95.2 (0.69)	94.7 (0.81)
P(HB-HV) film	94.9 (0.29)	89.9 (1.16)	89.5 (1.56)
PMMA film	93.8 (0.58)	** 60.5 (1.62)	**57.8 (0.66)
PAA film	** 18.9 (1.97)	** 0.00 (0.00)	** 0.00 (0.00)
HB 1mg/ml	100 (0.00)	98.6 (0.66)	99.1 (0.76)
2mg/ml	100 (0.00)	100 (0.00)	98.3 (0.78)
3mg/ml	97.9 (1.16)	97.5 (0.80)	97.5 (0.80)
5mg/ml	98.5 (0.70)	98.0 (1.05)	97.4 (1.08)
10mg/ml	99.3 (0.52)	97.0 (0.81)	96.7 (0.69)
15mg/ml	89.7 (0.23)	88.3 (1.21)	*84.3 (1.56)
20mg/ml	*80.1 (1.16)	**75.4 (0.69)	**49.5 (1.56)
25mg/ml	**48.7 (1.79)	**0.00 (0.00)	**0.00 (0.00)
35mg/ml	**0.00 (0.00)	**0.00 (0.00)	**0.00 (0.00)
HV 1mg/ml	98.6 (0.90)	98.1 (1.00)	98.1 (0.35)
2mg/ml	98.9 (0.90)	98.9 (0.44)	98.9 (0.44)
3mg/ml	98.2 (0.34)	98.7 (0.61)	98.7 (0.64)
5mg/ml	97.6 (0.69)	98.9 (0.31)	98.4 (1.06)
10mg/ml	97.4 (0.64)	97.9 (0.87)	98.8 (0.69)
15mg/ml	97.7 (0.52)	95.7 (0.69)	94.0 (0.48)
20mg/ml	*80.1 (1.16)	**75.4 (0.69)	**49.5 (1.56)
25mg/ml	**0.00 (0.00)	**0.00 (0.00)	**0.00 (0.00)
35mg/ml	**0.00 (0.00)	**0.00 (0.00)	**0.00 (0.00)
Sucrose			
60mg/ml	97.5 (0.42)	95.5 (0.03)	95.4 (0.77)
100mg/ml	94.6 (1.54)	89.6 (1.01)	88.6 (0.63)
NaCl			
12.5mg/ml	95.4 (0.46)	89.1 (0.69)	*75.8 (0.33)
25mg/ml	**0.00 (0.00)	**0.00 (0.00)	**0.00 (0.00)

\*p <0.01 Statistically significant at the 99% confidence interval when compared to the mean control value.

\*\*p <0.001 Statistically significant at the 99.9% confidence interval when compared to the mean control value. Student's t test for paired samples.



colonies PHB induced only a 5.3% colony death, whereas P(HB-HV) induced 10% colony death in the same period. 24 and 48hr exposure of these two polymers induced insignificant ( $p>0.05$ ) colony death when compared to the master replica disc. PMMA, of reported biocompatibility, induced significant ( $p<0.001$ ) colony death after 48 and 72 hrs exposure. PAA of reported cytotoxicity induced total colony death, i.e. cell death between 24 and 48hr exposure. The monomer, HB, was well tolerated at concentrations  $<15\text{mg/ml}$  however at concentrations above this significant ( $p<0.001$ ) colony death was seen. Similarly, HV caused significant ( $p<0.001$ ) colony death at concentrations above  $20\text{mg/ml}$  and this was thought to be an osmolarity effect. However,  $100\text{mg/ml}$  sucrose did not produce colony death as would be expected from the growth curve data, suggesting the possibility that colonies of cells require a greater source of sugar and therefore energy requirement to maintain growth than single cells in monolayer.

### 3.3. Discussion

Comparison of the population doubling times of treated cells with control cells can be used to estimate induced toxicity (Pappas and Cohen, 1968; Mital and Cohen, 1968; Rae, 1975, 1977, 1981). Population growth parameters give an indication of the rate of doubling of reproductively competent cells within that population and is a function of the number of cycling cells and their cycle time. Defective cellular reproduction is manifested by impaired growth and maintenance of the population of affected cells. It is known that defective population growth may be due to: 1) reproductive arrest 2) slowed cell cycling 3) unregulated cell cycling. A cell in reproductive arrest is one that has incurred damage resulting from

permanant blockage of cell cycle transit. The blockage can occur in various phases of the cell cycle, indicating that defects of different cellular processes may be responsible (Mauro and Madoc-Jones, 1970). However, it is important to realise the cell is not necessarily dead. Slowed population growth may be produced by temporary blockage of the cell cycle or by permanent slowing of cell cycle transit. Both responses often result from cellular defects that affect a specific event during one cycle phase. Unregulated proliferation i.e cycling may also occur and this has been equated to neoplastic transformation (Monat and Loeb, 1983). Reproductive arrest, slowed cell cycling and unregulated cycling can all occur through the toxic effects of chemicals and/or physical injury which may be imparted to the tissue by the implanted polymer.

The calculation of population doubling times obtained from the construction of growth curves for the copolymer, PMMA and monomer treated cultures compared to untreated cultures of CHO-K1 cells, suggested slowed cell cycling had occurred. Divergent slopes, seen with P(HB-HV) and PMMA (fig. 3.1.) and increasing concentrations of the monomers (fig. 3.4. and 3.5.) in the culture medium, when compared to the slope of the control culture has been suggested to indicate reversible toxicity which merely slows or transiently blocks cell proliferation (Painter, 1977). As both the soluble monomer and polymer films appeared to cause slowed cell cycling, it was believed that their physical presence (i.e as expected with the films) did not impede cell growth. The slowed cell cycling observed with the increasing concentrations of both monomers was believed to be due at least in part to an osmotic effect. Experiments to determine whether osmolarity affected the cell cycling of CHO-K1 cells indicated that high osmolarity of culture medium ( $>280\text{mmol}$ ) increased the population

culture medium cell death occurred. At no time was pH seen to affect cell growth as the sodium bicarbonate buffering system in the culture media maintained the pH between 7.2 and 7.6 even with increasing concentrations of the monomers. Further to interpretation of the growth curve data it has been proposed that parallel slopes of treated cells and control cells indicate that a fraction of cells have been permanently prevented from cycling either by cell death or reproductive arrest (Grisham and Smith, 1984). The fraction of dead or reproductively sterilized cells can be roughly estimated by extrapolation of the exponential portion of the curve to the cell axis. Parallelism of slopes was observed between P(HB-HV) treated CHO-K1 cells and PMMA treated CHO-K1 cells. The cells grown in the continued presence of copolymer and PMMA films show increased population doubling times, which could be due to the physical presence of the films or more probably, as the presence of PHB films did not similarly affect population growth, leaching of cytotoxic chemicals such as additives, plasticizers or their monomers. The deprivation of O<sub>2</sub> or nutrients was also unlikely to occur in the presence of the polymers films as the total area occupied at any one time was 36%. If osmolarity was not the cause of increased lag and population doubling times then to establish the critical adducts or the mechanism of cellular toxic response more sophisticated techniques would be required. However for the purpose of a rapid toxicological screening programme for biomaterials this would not be necessary.

Rae (1975, 1977, 1981), assessed the toxicity of particulate metals of orthopedic interest, using counts of cells in the lag phase of growth and in the replication phase of growth. It was thought the particulate presentation of the biomaterial would produce more

particulate presentation of the biomaterial would produce more sensitive results in cell culture due to the large surface area presented to the cells in monolayer. However problems of sedimentation, nonuniformity and problems of dispersion lead to difficulties in the exposure of the cells or colonies to identical doses. Therefore growth curve assessment of CHO cells was performed with their exposure to films of the polymers or solutions of their monomers.

The replica plating experiments measured the ability of colonies of cells to maintain growth i.e. the ability of the attached cells to proliferate and double with the required speed. If no toxic response occurs the established colony formation on the master replica disc should exactly match those of the treated discs. The cytotoxicity observed with the PMMA and PAA treated replica plates was believed to be chemically related. Leaching of cytotoxic materials used in the polymer preparation or its monomers was thought to induce colony death. The deprivation of O<sub>2</sub> and/or nutrients inducing colony death was not believed to occur as both polymers have a large number of cross-links in the structure allowing access of the O<sub>2</sub> and nutrients to the colonies.

The two cell culture methods have shown that PHB and its copolymer have good biocompatibility and that both the monomer products at 10-15 mg/ml concentrations are non toxic. This was in agreement with the data produced by Korsatko *et al.* (1984), where *in vitro* cellular growth and metabolism was measured for mice fibroblasts exposed to tablets of PHB.

The sensitivity of cell culture has allowed a rapid picture to be formed of a biomaterials acute toxic reaction and probable *in vivo* biocompatibility. However, there are disadvantages in relying solely

on the results from *in vitro* toxicity testing as *in vivo* cells rarely act in isolation since the tissue reaction to implanted materials is likely to be a series of complex, integrated responses which may take place over many months. Therefore the conditions reproduced *in vitro* are a very simplified part of what may occur *in vivo*. One other major disadvantage of cell culture is that toxicity *in vitro* may be an overestimate of the toxicity *in vivo* due to the relatively stagnant conditions of the system. Cells in culture are also isolated from important detoxification and excretion pathways. Furthermore the metabolism of the cells in culture is abnormal. A solution to this problem has been suggested by the use of liver homogenate preparations, such as the mammalian microsomal 'S9' mix (Styles, 1981). However this does not necessarily represent metabolism in non-hepatic tissues. These disadvantages do not preclude the use of *in vitro* cell culture as a toxicological screen, but used in conjunction with *in vivo* animal experiments the biocompatibility of new materials may be established with greater efficiency and sensitivity.

Tissue culture methods employed for mutagenicity testing and carcinogenicity testing in the assessment of polymer toxicity are also desirable but are beyond the scope of this present work.

### 3.4. Conclusions.

1) No significant difference ( $p > 0.05$ ) in the lag or  $T_{1/2}$  growth parameters of CHO-K1 cells grown in the continuous presence of PHB or P(HB-HV) films.

2) No significant difference ( $p > 0.05$ ) in the lag or  $T_{1/2}$  growth parameters of CHO-K1 cells pretreated for 48 hours with PHB or P(HB-HV).

3) At concentrations greater than 10mg/ml, HB caused an increase in both the lag time and  $T_{1/2}$  when compared to control cultures, and at concentrations greater than 25mg/ml cell death occurred.

4) At concentrations greater than 15mg/ml, HV also increased lag time and  $T_{1/2}$ .

5) Increase in osmolarity was thought to be the cause of the cytotoxicity as shown with experiments using NaCl and sucrose.

6) The second cell culture method to determine cytotoxicity and therefore biocompatibility gave results that agreed with the growth curve method, indicating the reproducibility of *in vitro* cell culture methods.

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## CHAPTER 4. The effect of PHB, P(HB-HV) and their degradation products on activation of the macrophage.

### 4.1. Introduction

Over the past decade a number of investigators have examined the effects of biomedical materials on macrophage accumulation *in vivo* and macrophage activation *in vitro* with regard to prediction of the material's biocompatibility (Miller *et al.*, 1989; Spilizewski *et al.*, 1987; Miller and Anderson, 1988; Marchant *et al.*, 1984 and Bakker *et al.*, 1988). Emphasis has been put on macrophage activation in biocompatibility studies as it plays a crucial part in the pathophysiology of inflammation, (Baggiolini, 1985) by virtue of its phagocytic capabilities and release of factors into the extracellular inflammatory exudate, (Schorlemmer *et al.*, 1977; Rae, 1975, 1981; Reiko *et al.*, 1984; Nathan, 1987). Dependent upon the concentration of macrophage accumulation and then release of these mediators, inflammation may be exacerbated or resolved (Ziats *et al.*, 1988). An understanding of the interaction between macrophages and the foreign material may lead to a prediction of the reaction of tissues to the implantation of biomaterials.

Macrophage activation has been defined by a number of workers (Petty, 1978; Lasser, 1983; Davies *et al.*, 1974) as an increase in functional activity induced by a given stimulus when compared to the resident macrophages which are present at any given site in the absence of an exogenous or endogenous inflammatory stimulus. In many instances macrophage activation has been said to accompany or precede the process of phagocytosis and also the subsequent release of inflammatory mediators into the area of stimulus (Henson, 1980; Anderson *et al.*, 1984; Synderman *et al.*, 1971).

During macrophage activation the macrophage undergoes many



changes with production and release of a myriad of substances including oxidised products of arachidonic acid through the cyclooxygenase cycle, of which prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is the major end product (Davies *et al.*, 1980; Scott *et al.*, 1980; Passwell *et al.*, 1979 and Humes *et al.*, 1977; Artursson *et al.*, 1987). PGE<sub>2</sub> has therefore been used as an index of phagocytic activation by many investigators in the assessment of biomaterial toxicity (Miller *et al.*, 1989; Elliott *et al.*, 1987).

Macrophage activation has been investigated *in vitro* using cultures of freshly isolated mouse peritoneal macrophages, (Parwaressch *et al.*, 1981; Schorlemmer *et al.*, 1976, 1977; Pizzoferrato *et al.*, 1987 and Raz *et al.*, 1977). A variety of different biological endpoints/parameters have been determined including the production and release of PGE<sub>2</sub> and lysosomal enzymes (Cohn *et al.*, 1963; Cardella *et al.*, 1974; Allison *et al.*, 1975; Axiline *et al.*, 1970 and Weissman, 1971). Lactate dehydrogenase (LDH) release has been used as a marker of cytotoxicity of these cells by a number of workers, (Chambers, 1981; Weissman *et al.*, 1971) and may also be used in the evaluation of the biocompatibility of implant materials. Changes in cellular morphology have also been used as an indicator of macrophage activation (Leake *et al.*, 1975, 1979; Rasp *et al.*, 1979 and Raz *et al.*, 1977; Becker *et al.*, 1986; Miller *et al.*, 1989). The latter include the measurement of the amount of membrane ruffles and the extent of cell attachment and spreading as compared to the same parameters in non-activated cells.

In the following chapter work will be presented showing the effect of PHB, P(HB-HV) and their degradation products on the kinetics and accumulation of macrophages *in vivo* and the extent of activation of resident and elicited macrophages *in vitro*. A known macrophage

activator, opsonized zymosan, (Schnyder and Baggiolini, 1978; Bonney *et al.*, 1978; Henson, 1980 and Humes *et al.*, 1977) was used as a positive control in this study. Macrophage activation was assessed *in vitro* by the release of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in culture. Cytotoxicity was assessed by LDH production and release. Morphological changes and the extent of phagocytosis shown by macrophages in culture, when activated by certain materials, were observed using a scanning electron microscope.

#### 4.2. Results

According to the sterility assay (B.P. 1988 Vol. II, appendix XVI) and the Limulus amoebocyte lysate (LAL) assay for endotoxins (B.P. 1988 Vol. II, appendix XIV and using Sigma procedure 210-2) suspensions of polymers and solutions of their monomers were determined to be essentially free of bacterial contaminants and endotoxins thereby ensuring any macrophage activation was due to the biomaterials.

Four days following intra-peritoneal injection (as described in 2.2.2), the number of cells classified as macrophages when examined by light microscopy after staining with Giesma stain was found to be ~ 85%. Typical characteristics of macrophages observed after treatment with Giesma included staining of a kidney-shaped or bi-lobed nucleus surrounded by a relatively large volume of cytoplasm which remained uncoloured. Their typical diameters ranged from 15 to 20  $\mu\text{m}$  compared to the smaller PMN's 8  $\mu\text{m}$ . Following the period of 3 hrs allowed for adherence to the culture plates or glass coverslips then subsequent washing off of nonadherent cells, the macrophage population was determined to be >95%. The viability as indicated by phase contrast microscopy after 24 hrs culture was > 90%.

##### 4.2.1. Quantification kinetics of macrophage accumulation *in vivo*.

Table 4.1. shows the macrophage number accumulated in the peritoneal cavity following injection with the test materials. To provide a comparative index for macrophage accumulation and activation, thioglycollate media, a known eliciting agent of macrophages, and opsonized zymosan, a known macrophage activator, were used as positive controls. Intraperitoneal injection of PBS (pH

Test material (100mg/ml)	Days					
	2	4	6	8	10	28
	Macrophage accumulation $\times 10^6$ mean (S.D.) n=4					
PHB	2.10 (0.31)	4.55 (0.26)	12.75 (0.84)	11.30 (2.10)	4.51 (0.62)	1.40 (0.10)
P(HB-HV)	2.55 (0.20)	3.35 (0.53)	4.12 (0.53)	4.20 (1.50)	3.03 (0.48)	1.30 (0.11)
HB	1.04 (0.03)	0.91 (0.09)	2.27 (0.25)	2.89 (0.25)	2.23 (0.2)	1.46 (0.08)
HV	1.39 (0.03)	1.57 (0.17)	2.40 (0.14)	2.82 (0.30)	2.43 (0.20)	1.34 (0.11)
PBS (negative control)	0.90 (0.00)	0.85 (0.20)	2.37 (0.21)	1.66 (0.21)	2.18 (0.25)	1.20 (0.11)
PMMA (negative control)	1.13 (0.09)	1.39 (0.12)	2.25 (0.17)	2.52 (0.39)	1.77 (0.25)	1.40 (0.24)
Chalk (positive control)	3.47 (0.49)	3.08 (0.09)	2.42 (0.17)	2.43 (0.16)	4.83 (0.18)	1.72 (0.12)
Thioglycollate (positive control)	2.20 (0.24)	1.74 (0.12)	4.20 (0.24)	2.94 (0.12)	2.56 (0.21)	1.30 (0.12)
Zymosan (100ug/ml) (positive control)	2.50 (0.28)	5.10 (0.18)	14.8 (1.00)	18.00 (0.32)	11.4 (0.69)	5.20 (0.33)

Table 4.1. :Quantification of the kinetics and magnitude of macrophage accumulation *in vivo*  
Figures are mean (S.D.) of 4 experiments.

= 7.3) with 1% methylcellulose, the injection vehicle, was used as the negative control. The results showed good reproducibility for macrophage accumulation for all materials tested (s.d. <15%) between animals.

Injection of PHB particles into the peritoneal cavity of CLFP mice resulted in an increase of macrophage accumulation over an 8 day period which gradually decreased to vehicle control macrophage levels. At day 4 there was a significant increase in macrophage number ( $p < 0.01$ ) when compared to the vehicle, and at days 6 - 8 a highly significant increase ( $p < 0.001$ ) in macrophage number was seen. Zymosan resulted in a highly significant increase ( $p < 0.001$ ) in macrophage numbers from days 4 - 28 when compared to vehicle control groups. Thioglycollate induced a significant increase ( $p < 0.01$ ) in macrophage numbers at days 6 and 8 only and the counts were considerably lower than those produced by PHB or zymosan particles.

P(HB-HV) resulted in a significant increase ( $p < 0.01$ ) in macrophage number when compared to the vehicle control on day 6, followed by a decrease to vehicle control macrophage numbers by day 28. The degradation products of the two polymers, HB and HV, resulted in significant increases ( $p < 0.01$ ) in macrophage number at day 8. Significant increases were thought to be indicative either of inflammation and toxicity or macrophage activation and release of factors which attracted more macrophages to the site of injection.

In summary, when compared to the resident macrophage population in freshly killed CLFP mice of  $0.95 \times 10^6 \pm 0.12$ , PBS with 1% methylcellulose causes little increased accumulation following peritoneal injection. HB, HV, PMMA and chalk had no significant effect on macrophage accumulation. Thioglycollate, a known eliciter of macrophages, and P(HB-HV) showed only a slight increase in macrophage accumulation number following their respective

injections. PHB and zymosan had a significant effect on macrophage accumulation on days 4-10.

#### 4.2.2. Effect of particulate concentration on macrophage accumulation.

Table 4.2. shows that over the ten day period the number of macrophages attracted to the peritoneal cavity after injection with PHB microspheres was significantly greater ( $p < 0.01$ ) for the 100mg/ml injection when compared to the 50 mg/ml injection. This suggested a dose response to the materials in terms of the numbers of elicited macrophages. This could be explained by : i) a greater inflammatory stimulus therefore attracting more macrophages to the area or ii) a greater number of particles liable to be phagocytosed thereby attracting more macrophages at the intra-peritoneal site. It was interesting to note that the number of macrophages attracted to the site of PHB injection (100mg/ml) was  $\sim x6$  greater on day 6 and  $x4$  greater on day 8 than numbers attracted by the equivalent concentration of chalk powder. This effect may only in part be due to differences in the surface characteristics of the materials since at 50mg/ml the PHB microspheres and chalk powder did not significantly differ in the number of macrophages attracted to the site of injection. P(HB-HV) (100mg/ml) caused an approximate doubling in macrophage numbers on days 6 - 8 when compared to the equivalent concentration of chalk powder, but at 50mg/ml the P(HB-HV) microspheres and chalk powder did not significantly differ in the number of macrophages attracted to the site of injection.

#### 4.2.3. Effect of biomaterials on the activation of the macrophages *in vitro*.

Table 4.3. shows the mean concentration of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)

		Days				
Test material		2	4	6	8	10
		macrophage accumulation x10 <sup>6</sup> mean (S.D.) n=4				
PHB	50mg/ml	1.37 (0.20)	2.39 (0.23)	5.30 (0.33)	1.98 (0.37)	3.02 (0.27)
	100mg/ml	2.10 (0.35)	5.04 (0.42)	13.80 (0.59)	9.15 (0.37)	4.67 (0.45)
P(HB-HV)	50mg/ml	1.82 (0.12)	2.49 (0.31)	2.40 (0.21)	2.31 (0.19)	1.79 (0.14)
	100mg/ml	2.30 (0.22)	3.57 (0.21)	4.30 (0.29)	4.10 (0.31)	3.13 (0.27)
Chalk powder	50mg/ml	1.66 (0.18)	2.25 (0.19)	1.89 (0.15)	1.80 (0.19)	0.96 (0.09)
	100mg/ml	2.77 (0.24)	3.37 (0.27)	2.31 (0.2)	2.37 (0.11)	4.78 (0.22)

Table 4.2.:- The effect of particulate concentration on the extent of *in vivo* macrophage accumulation.  
 Figures are mean (S.D.) of 4 experiments.

Test material	Time									
	mins					hours				
	1	3	5	10	15	1	2	3	5	24
	Measurement of PGE <sub>2</sub> release in pg/ml mean (S.D) n=3									
HB (100mg/ml)	544 (33)	984 (71)	1027 (43)	990 (9)	540 (36)	410 (50)	557 (93)	130 (74)	178 (23)	207 (33)
HV (100mg/ml)	917 (62)	1010 (12)	1000 (87)	1035 (21)	532 (36)	350 (71)	485 (29)	567 (119)	347 (29)	282 (21)
P(HB-HV)(10mg/ml)	583 (164)	560 (57)	680 (152)	703 (104)	580 (73)	603 (156)	795 (138)	737 (21)	240 (29)	187 (54)
P(HB-HV)(100mg/ml)	857 (47)	983 (132)	1023 (29)	907 (137)	637 (90)	622 (170)	727 (35)	410 (121)	463 (159)	157 (31)
PHB (10mg/ml)	648 (74)	1010 (31)	1130 (128)	1030 (16)	220 (62)	526 (47)	590 (38)	528 (24)	466 (114)	102 (14)
PHB (100mg/ml)	583 (123)	855 (74)	887 (126)	1050 (42)	517 (78)	737 (47)	798 (157)	737 (97)	536 (45)	188 (24)
Control media	95 (16)	88 (19)	93 (26)	105 (5)	75 (16)	68 (19)	85 (26)	65 (24)	107 (17)	85 (10)
Zymosan (100ug/ml)	797 (38)	1150 (107)	1350 (254)	1050 (45)	683 (81)	960 (69)	860 (57)	838 (36)	748 (43)	683 (67)

Table 4.3. : The effect of biomaterials on the production of PGE<sub>2</sub> in macrophages *in vitro*.  
Figures are mean (S.D.) of 3 experiments.



Test material	Time									
	1	3	5	10	15	1	2	3	5	24
	← mins →					← hrs →				
HB (100mg/ml)	5.83	11.14	11.00	9.43	7.20	6.00	6.55	2.00	1.67	2.43
HV (100mg/ml)	9.65	11.44	10.72	9.86	7.08	5.12	5.71	8.72	3.25	3.31
P(HB-HV) 100mg/ml)	9.02	11.14	10.96	8.63	8.49	9.10	8.55	6.31	4.34	1.84
PHB (100mg/ml)	6.14	9.68	9.50	10.00	6.89	10.79	9.39	11.33	5.03	2.22
Zymosan (100 $\mu$ g/ml)	8.38	13.02	14.50	10.00	9.10	14.06	10.12	12.90	7.01	8.03

Table 4.4. : Ratio of test PGE<sub>2</sub> production for macrophages *in vitro*.  
control

The control value was taken as the amount of PGE<sub>2</sub> measured in the media of control cultured macrophages at specific time intervals.

The ratio was calculated using mean of 3 values.

released from macrophages following incubation with each of the test polymers or their monomers. The culture medium and tissue culture grade plastic dish was used as the negative control and the opsonized zymosan as the positive control.

There was a highly significant increase ( $p < 0.001$ ) in PGE<sub>2</sub> production in the first 15 minutes of culture following treatment with all polymers and monomers compared to the negative control. From 1 hr to 24 hrs in culture there was a gradual decrease in PGE<sub>2</sub> production with all polymers and monomers. The opsonized zymosan showed a significant increase ( $p < 0.001$ ) in PGE<sub>2</sub> production and release throughout all time intervals in the 24 hr culture with only a small decrease in activation after 24 hrs.

The results are summarized in table 4.4. which expresses the extent of macrophage activation as a ratio of PGE<sub>2</sub> production from test material treated macrophage cultures and untreated macrophage cultures.

#### 4.2.4. Cytotoxicity as shown by the LDH assay.

An increase in LDH release into culture media occurs on lysis of macrophages and this phenomenon has been used to measure cellular toxicity and hence predict the material's biocompatibility. Table 4.5. shows significant increases in LDH concentration in the media for both HB and HV treated cultures, but only a small insignificant increase ( $p > 0.05$ ) at the end of the 24hr culture period for the other materials. It was assumed that macrophage lysis and therefore cellular incompatibility occurs only at high concentrations of degradation products and that the polymers do not therefore adversely affect macrophages.

#### 4.2.5. Scanning electron microscopy.

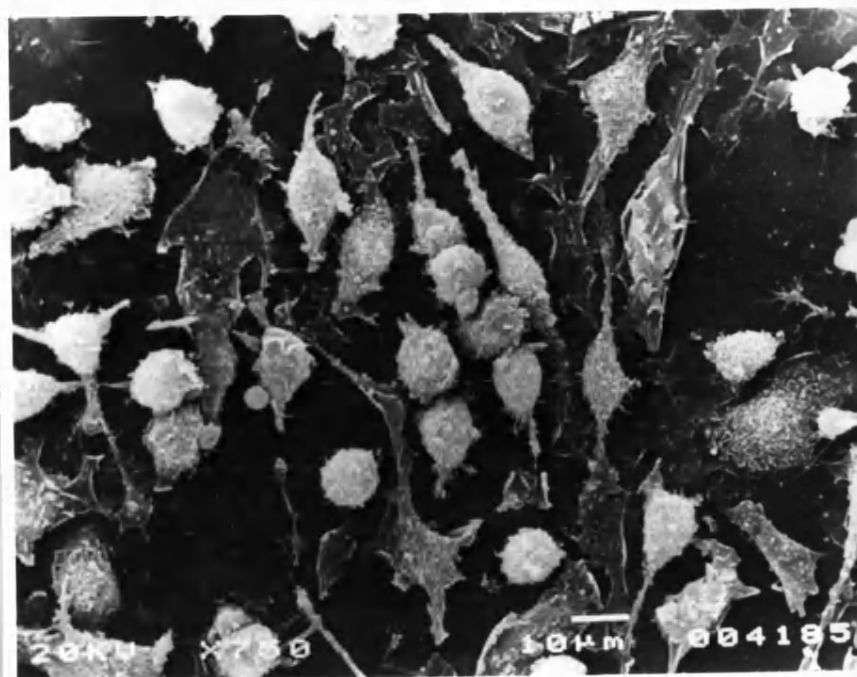
Test materials	10	15	30	1	2	3	5	24
	← mins →			← hrs →				
	LDH production in D/D per ml mean (S.D.) n=3							
HB (100mg/ml)	380 (51)	430 (43)	380 (37)	1168 (241)	657 (95)	1113 (471)	1419 (578)	1483 (346)
HV (100mg/ml)	460 (56)	530 (86)	1865 (149)	1868 (149)	2273 (117)	1970 (110)	2120 (36)	1593 (624)
P(HB/HV) (10mg/ml)	0.0	0.0	74 (105)	276 (39)	487 (114)	627 (95)	857 (91)	2140 (110)
P(HB/HV) (100mg/ml)	0.0	0.0	0.0	0.0	213 (26)	558 (48)	1163 (144)	2033 (343)
PHB (10mg/ml)	0.0	0.0	0.0	487 (135)	637 (76)	710 (123)	767 (99)	870 (104)
PHB (100mg/ml)	1283 (284)	550 (51)	357 (63)	644 (300)	720 (173)	707 (164)	783 (237)	1510 (387)
Control Media	355 (23)	563 (120)	617 (246)	1057 (107)	531 (106)	682 (71)	704 (110)	597 (113)
Zymosan (100µg/ml)	387 (24)	303 (66)	303 (21)	317 (50)	327 (56)	300 (24)	336 (47)	497 (86)

Table 4.5. The effect of biomaterials on LDH production in *in vitro* macrophage cultures.

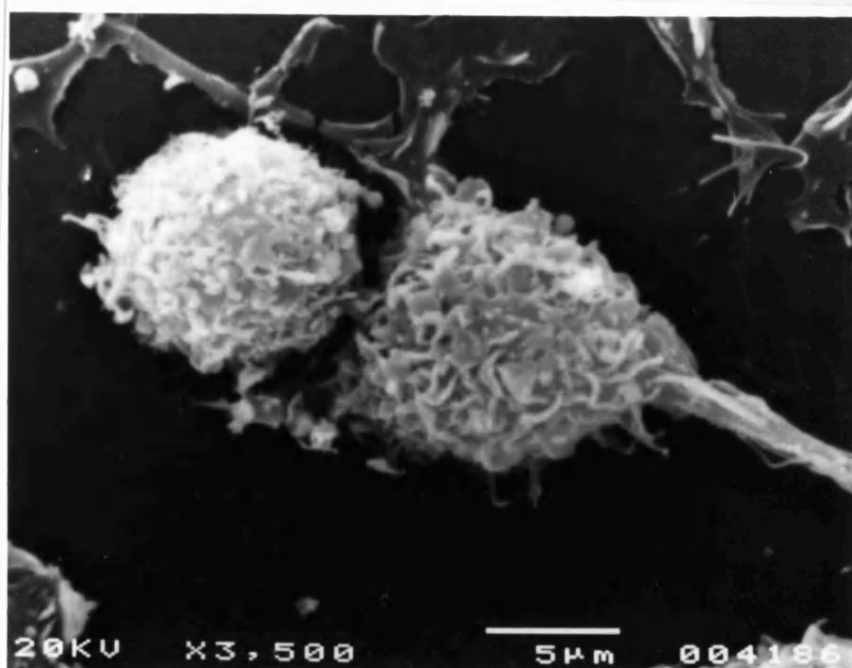
Studies using scanning electron microscopy revealed that the surfaces of the macrophage population cultivated with 100mg/ml of PHB suspended in RPMI 1640 media, possessed a large number of ridges or ruffles. ( Figures 4.1. and 4.2.). The electron micrographs show little cellular spreading at the membrane periphery i.e. few filopodia, however the cells themselves had adopted an elongated profile. The large numbers of ruffles and elongation of the macrophages were thought to be indicative of activation.

P(HB-HV) caused similar morphological effects in the macrophages (figures 4.3. and 4.4.), but monomer (100mg/ml) treated macrophage slight elongation in morphology but with little evidence of membrane ruffles. The majority of the monomer treated macrophages appeared to be attached to the glass coverslip by long thin filopodia at one end of the cell (figure 4.5.).

Normal macrophages showed little ruffling of the membrane, a smooth peripheral membrane with little filopodia at the periphery and little cellular spreading (figures 4.6.).



4.1

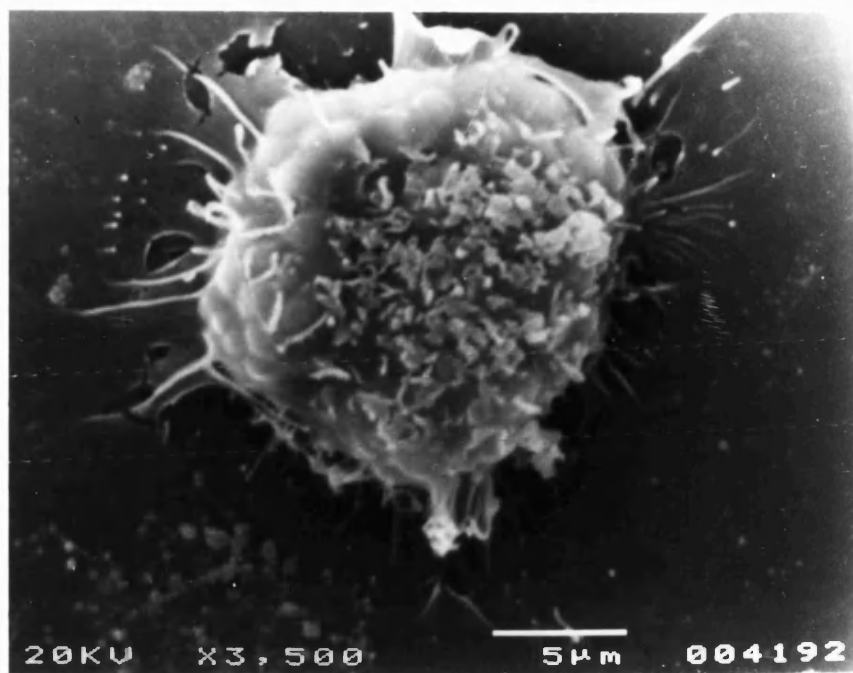


4.2

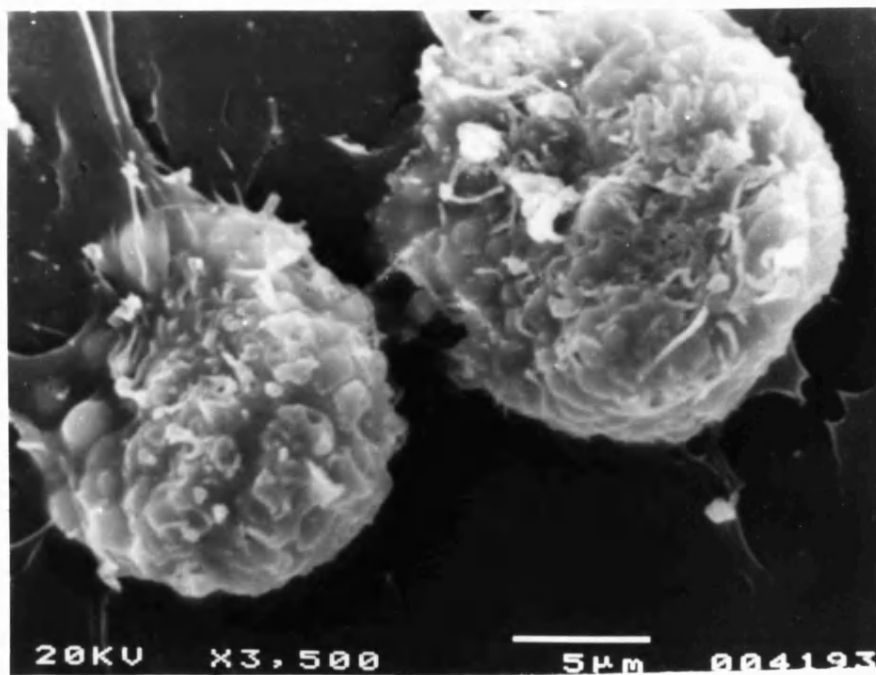
Figures 4.1 and 4.2. Scanning electron micrographs of macrophage cultures treated with PHB(100mg/ml) cultivated for 15 hours in RPMI 1640 medium containing 10% v/v heat inactivated foetal calf serum.

4.1) x 750 20KV

4.2) x 3500 20KV

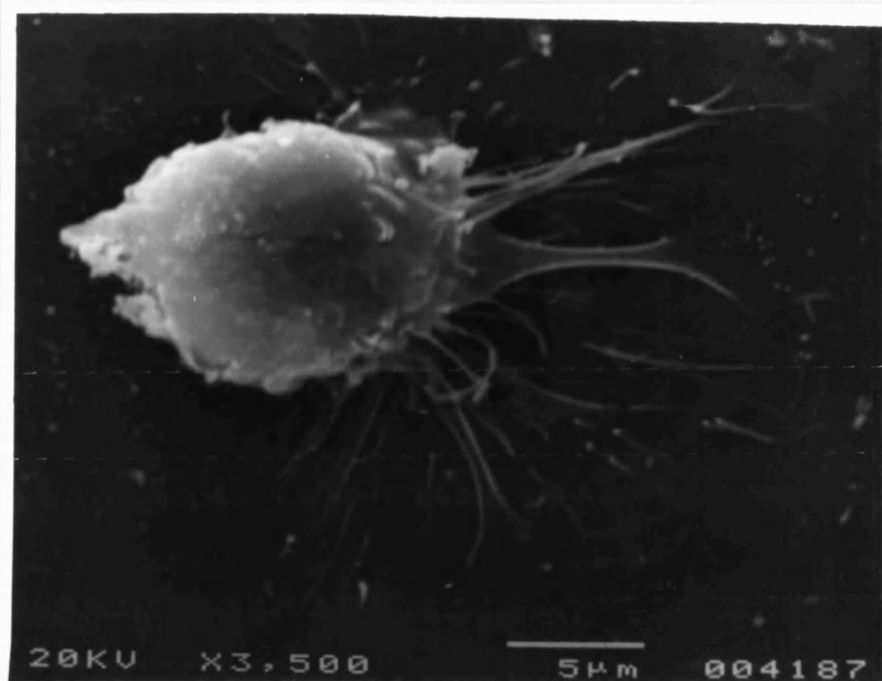


4.3

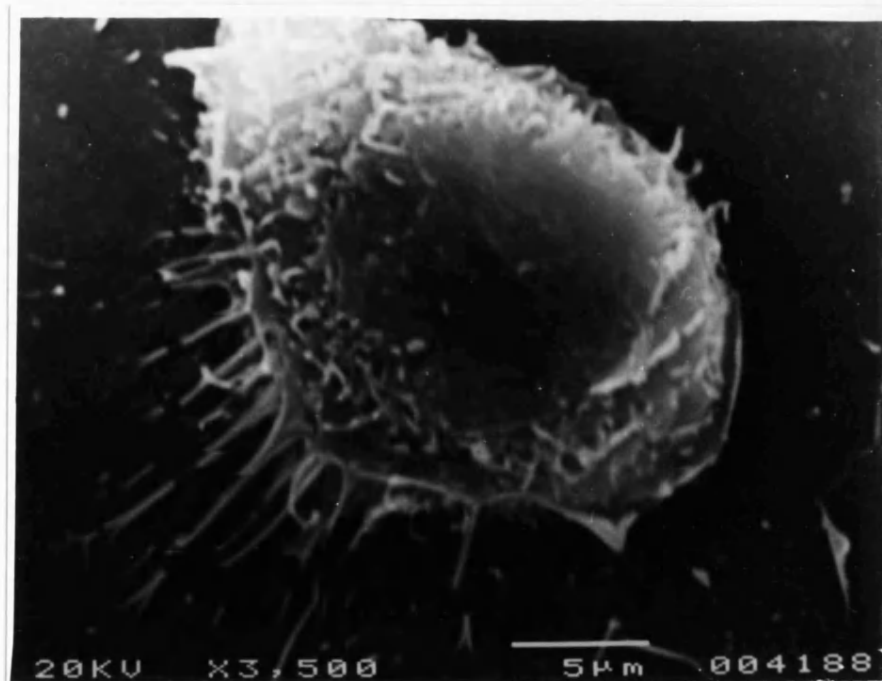


4.4

Figures 4.3 and 4.4. Scanning electron micrographs of macrophage cultures treated with P(HB-HV) 100mg/ml, cultivated for 15 hours in RPMI 1640 medium containing 10%v/v heat inactivated foetal calf serum. X 3500 . 20KV



4.5



4.6

Figure 4.5. Scanning electron micrograph of macrophage culture treated with 100mg/ml HB, cultured for 15 hours in RPMI 1640 medium containing 10%v/v heat inactivated foetal calf serum. x3500 20KV.

Figure 4.6. Scanning electron micrograph of 'normal' macrophages in culture. x3500, 20KV.

### 4.3 Discussion

The mononuclear phagocytic system is a line of cells that originate in the bone marrow, are transported by the blood stream and localize in the tissues where they differentiate to form macrophages. Macrophages have globulin receptors, complement C3 receptors and receptors for the Fc portion of IgG with three different Fc expressions on their surfaces, (Rabinovitch 1970 and Lasser *et al.*, 1982; Gordon and Rabinowitz, 1989). During inflammation the mononuclear phagocytes are chemotactically attracted to the inflammatory focus by a number of mediators including complement components (Boyden, 1972 and Schorlemmer *et al.*, 1976; Mellman *et al.*, 1988) and antigen antibody complexes (Walker, 1977 and Steinman, 1974; Steinman *et al.*, 1983). The number of macrophages attracted to the site of inflammation is dependent on the size of the inflammatory stimulus, (Scott *et al.*, 1980 and Petty, 1978). In this study the greatest polymer induced accumulation of macrophages occurred on days 6-8 after mice were injected intraperitoneally with PHB microspheres. This could be explained either by toxicity of the material which would be reflected in the magnitude and duration of the inflammatory response (see chapter 8 for a discussion of the role of macrophages in inflammation), or an attempted phagocytosis of PHB without any toxicity to the tissues. The latter conclusion was favoured as no necrosis or chronic inflammation was obvious at the site of injection, as verified by the histology, vascular permeability and enzyme histochemistry work described in chapter 6.

Thioglycollate is a known macrophage 'eliciter' (Gordon, 1976 and Conrad, 1977) and was used as a positive control in the *in vivo* studies of accumulation of macrophages after intraperitoneal injection. The effect of thioglycollate was to cause a doubling of the count on day 6 when compared to the vehicle control and slightly



raised counts on other days. Zymosan also a positive control, and at a tenth (100ug/ml) of the concentration of polymers injected in this experiment, was seen to induce a much greater increase in accumulation throughout the study, reaching a peak on day 8 which was sustained until day 28. PHB caused a comparable accumulation on day 6 but the levels had returned to normal by day 28. Clearly the effect of PHB was short-lived in comparison with zymosan. This could be explained in part by ongoing phagocytosis of the zymosan particles (~3um in diameter) and therefore the subsequent attraction of more macrophages to this inflammatory stimulus. PHB however, initially attracts the macrophages to its site of injection in order to be phagocytosed, but because the particle size is 40 um in diameter, (~x3 bigger than the actual macrophage) 'frustrated' phagocytosis may occur. As the polymer is non-toxic to the tissues the macrophage number decreases. The accumulation of macrophages after injection of P(HB-HV) was similar to the accumulation after injection of thioglycollate (table 4.1.), the macrophage accumulation number only significantly greater ( $p < 0.01$ ) at day 6 when compared to the vehicle control. The differences in macrophage accumulation number of PHB and P(HB-HV) were believed to be due in part to the difference in surface chemistry of the two polymers. The dissolved monomers had little effect on macrophage activation *in vivo* which was expected as the monomers would be cleared rapidly from the peritoneal cavity.

Following chemotaxis to the inflammatory stimulus, depending on the material (solution or particulate), phagocytosis or pinocytosis (fluid-phase endocytosis) can occur. Pinocytosis of small volumes of fluid is likely to be constitutive whereas phagocytosis in many cases requires activation of the macrophage: i.e. the macrophage undergoes several morphological, biochemical and functional changes when expressing enhanced resistance to foreign material and

inflammatory stimuli (Nathan, 1987; Mackanass, 1964, 1970; Macmonie *et al.*, 1968 and Humes *et al.*, 1977).

Over 50 structural and biochemical alterations have been shown to occur in activated macrophages, some of which are listed in table 4.6. The measurement of the production and release of PGE<sub>2</sub> from activated macrophages is particularly useful as PGE<sub>2</sub> plays a key role in mediating inflammatory reactions and also participates in the regulation of immune functions (Passwell *et al.*, 1979). The critical role of PGE<sub>2</sub> in the inflammatory process is suggested by the fact that the tissue concentration of prostaglandin E<sub>2</sub> correlates with the local accumulation of the inflammatory exudate and inhibitors of prostaglandin synthesis decrease the intensity and duration of inflammation (Vane, 1971, 1976 and Flower, 1976). *In vitro* studies have shown that PGE<sub>2</sub> also inhibits immune function in general (Bray *et al.*, 1978) and inhibits macrophage inhibitory factor production (Gordon *et al.*, 1976) thereby exacerbating the inflammatory process.

During macrophage phagocytosis and activation the magnitude of prostaglandin synthesis and duration of production is determined by the phagocytic stimulus. As shown in this study the greatest increase in PGE<sub>2</sub> synthesis was induced by opsonized zymosan, a known macrophage activator. Both PHB and P(HB/HV) induced immediate production and release of PGE<sub>2</sub> within the first 15 minutes of culture indicating an initial macrophage activation, however this production and release decreased steadily over the 24 hr culture period whereas the activation caused by the zymosan was potentiated. This pattern of events could be explained by : 1) an initial macrophage activation and release of PGE<sub>2</sub> associated with attempted phagocytosis of the particles, and 2) followed by an inhibition feedback system caused by down-regulation of the stimulation of

Table 4.6. Characteristics of activated macrophages.

Morphological increases in:

Size  
adhesiveness and spreading  
Ruffled membrane activity  
Cytoplasmic granules

Biochemical increases in:

Metabolic activity  
Adenyl cyclase  
Cyclic GMP  
Calcium ion influx  
Glucose oxidation  
Lysosomal enzymes and their release  
Lactic dehydrogenase  
Lysosyme  
Collagenase  
Elastase  
Plasminogen activator production  
Prostaglandin production  
Interferon

Functional increases in:

Pinocytosis  
Phagocytosis  
Intracellular microbicidal activity  
Cytotoxic effects on tumour cells

PGE<sub>2</sub> on macrophage activation resulting in less subsequent release of prostaglandin. This has been described by some investigators, (Baggiolini, 1985 and Passwell *et al.*, 1979; Elliott *et al.*, 1987). Schnyder *et al.*, 1978, 1980 and 1981 suggested that PGE<sub>2</sub> may counteract other activating agents and the amounts of PGE<sub>2</sub> produced in response to other activators determine the degree of macrophage activation. The down-regulation of activation was less pronounced after injection of zymosan particles.

Lactate dehydrogenase production is increased during macrophage activation, but LDH is only released when cell lysis occurs, (Henson, 1980; Chambers, 1981). LDH measurement was therefore used as an indication of the cytotoxicity of the biomaterials and their degradation products. In most cases the release of LDH from macrophages inoculated with the test samples was no different to the LDH measured in the media of control cultures. Significant ( $p < 0.01$ ) amounts of LDH were measured after the cultures were treated with HB and HV, particularly those treated with HV at 100mg/ml, indicating that the degradation products were cytotoxic at these concentrations. This cytotoxicity was comparable to that observed during cell culture work with CHO-K1 cells, and was thought to be a function of the osmolarity of the injection. The secretion of this enzyme by the macrophage indicates cytotoxicity and at the same time may exacerbate tissue damage leading to chronic inflammation as generation of inflammatory mediators then recruit more inflammatory cells to the focus. However, as described in chapter 6, when high concentrations of monomer (100mg/ml) were injected i.m. into rats no chronic inflammation was seen; this was probably due to the rapid clearance of the soluble monomers away from the site of injection, whereas *in vitro* the conditions are stagnant and therefore an artificial measure

of the monomer's toxicity is obtained.

SEM of cultured normal and polymer/monomer treated macrophage cultures revealed that the surface morphology of the latter appeared to differ significantly when compared to the former. The morphological features of the PHB and monomer treated macrophages were characterised by active cellular spreading indicated by the extension of long, thin filopodia and in the case of the polymer particles a highly ridged/ruffled membrane. It is generally believed that increases in the adherence of macrophages as indicated by active cellular spreading facilitate phagocytosis (Rabinovitch, 1970; Miller and Anderson, 1988). As no particle uptake was observed by light or scanning electron microscopy, this appearance of activation could be due to attempted or "frustrated" phagocytosis. Attempted phagocytosis has been described by a number of workers as a function of the size of particle or substrate (Lord, 1986) or the surface charge and hydrophobicity of the particulates or substrates, (Jones, 1975 and Wilkinson, 1974). Pizzoferrato *et al.* 1987 observed that the finer the particle size the greater the amount phagocytosed and transported. Surface charge is important in determining the acceptability of particles for ingestion. A particle with a strong net charge either positive or negative is ingested more avidly than an uncharged or weakly charged particle (Hench, 1982). Nishimura *et al.*, (1986) looked at macrophage activation with beads prepared from deacetylated chitin (a potential biodegradable drug delivery system). He postulated that before activation could take place contact with the surface of the material has to be made. This contact would occur with hydrophillic surfaces but not hydrophobic. As PHB and P(HB-HV) are more hydrophobic than zymosan particles, then the surface hydrophobicity could therefore partly explain why zymosan 1)

attracted more macrophages to the site of i.p. injection and 2) induced greater activation *in vitro*.

The parallelism between the capacity of various agents to induce chronic inflammation *in vivo* and the selective release of lysosomal enzymes and PGE<sub>2</sub> of peritoneal macrophages in culture is further discussed in chapter 8.

#### 4.4. Conclusions

1) Injection of PHB particles into the peritoneal cavity of CLFP mice resulted in highly significant increases ( $p < 0.001$ ) in macrophage accumulation number on days 6-8, which was comparable to injection of zymosan (a known macrophage eliciter) over 28 days. P(HB-HV), HB, and HV resulted in significant increases ( $p < 0.01$ ) in macrophage accumulation number on days 6 and 8, the numbers comparable to injection of thioglycollate media (another macrophage eliciter).

2) There appeared to be a dose response to the polymer materials in terms of numbers of elicited macrophages.

3) Macrophage activation, as indicated by PGE2 production and release appeared to increase significantly ( $p < 0.001$ ) in the first 15 minutes of macrophage culture following treatment with PHB, P(HB-HV) and their monomers when compared to the control macrophage culture.

4) Macrophage cytotoxicity, as indicated by significant increases ( $p < 0.01$ ) in LDH production and release, occurred at high concentrations of both monomers (100mg/ml). The effect was believed to osmotic.

5) Scanning electron microscopy revealed highly ruffled surfaces indicative of macrophage activation for polymer treated cultures only.

## CHAPTER 5. *In vitro* plasma protein and platelet interactions with PHB and P(HB-HV) in assessment of their blood compatibility.

### 5.1 Introduction

The performance of a biomaterial within the cardiovascular system is dependent on the manner in which the material interacts with the blood. An obvious requirement for such a biomaterial in contact with blood is its blood compatibility. Blood compatible materials have been broadly defined as those which do not cause thrombosis, destruction of the formed elements of the blood, alterations of plasma proteins, destruction of enzymes, or depletion of electrolytes (Williams, 1986).

For the design of experimental protocols to evaluate the blood compatibility of biomaterials, an understanding of the events preceeding thrombosis formation is required. Many investigators have found that when a foreign surface comes into contact with blood, one of the first events which occurs is the adsorption of plasma proteins (Baier, 1971,1977; Vroman, 1971; Lyman and Kim, 1971, Lyman, 1974). After this very rapid event some conformational and compositional rearrangement of the protein layer occurs which may be followed by platelet adhesion (Ito *et al*, 1989; Andrade, 1985; Morrissey, 1977; Lyman, 1975). At this stage the platelets may become activated, releasing a myriad of metabolically active products such as ADP and platelet factor 4, which cause further adhesion and aggregation of platelets, (Wintrobe, 1981; Fass *et al.*, 1978 and Weinstein, 1979). Accumulation of platelets can lead to a mural thrombus formation, which may be accompanied by fibrin formation following surface activation of the coagulation system. A summary of the events occuring at the blood-biomaterial interface is given in figure 5.1.



Instantaneous  
adsorption of some plasma proteins  
water and small ionic components.



Time-dependent  
desorption and enzymatic modification  
of adsorbed plasma proteins.



PLASMA COAGULATION

Fibrinogen - Fibrin.



PLATELETS

Adhesion, release, aggregation  
red and white cells.



"RED" THROMBUS

chiefly red cells  
in fibrin

(low shear)

(high shear)



"WHITE" THROMBUS

chiefly platelets  
in fibrin



FIBRINOLYSIS



BLOOD COMPATIBILITY

Figure 5.1. Behavior of synthetic materials in flowing blood.

(Reproduced from Bruck, S.D., Biomater., Med. Dev., Artif. Organs, 6, 341, 1978)

The character of the adsorbed protein layer has been shown to influence the subsequent fate of the biomaterial surface. Almost exclusively, fibrinogen and gammaglobulin have been found to enhance the adhesion of platelets to the surface (Whicher and Brash, 1978; Kim and Lee, 1979) while albumin passivates the surface and retards extensive thrombosis, (Young *et al.*, 1982; Ishikawa *et al.*, 1984). The blood however does not dictate the whole reaction; the physical, chemical and morphological properties of the biomaterial surface play an important part in the reaction. The most researched properties of the biomaterial surface in this respect are surface charge (Salzman, 1972 and Nossel *et al.*, 1969) surface tension and wettability of the surface (Jayakumari, 1985; Absolom *et al.*, 1987; Steinberg *et al.*, 1989).

The most common method of investigating blood or blood component interactions with biomaterials is *in vitro*. These experiments are often much easier, faster and less expensive to perform than *in vivo* tests. The test parameters are easier to control and the results more quantifiable. These advantages have resulted in widespread use of *in vitro* techniques to establish biomaterial blood compatibility although a main disadvantage is that the blood is usually anticoagulated and not tested under conditions resembling clinical situations.

The pivotal role of plasma proteins, in the interaction of blood with artificial surfaces, has prompted many investigators to devise techniques to measure the adsorption of certain plasma proteins to materials. Earlier techniques used to study the kinetics of protein-material interactions included circular dichroism (McMillan and Walton, 1974), electron microscopy (Valantine, 1959), infrared internal reflection microscopy (Brash and Lyman, 1969),

ellipsometry (Vroman, 1964), and in situ radioiodination of adsorbed protein followed by electrophoresis (Horbett and Weathersby, 1981). In more recent years radiometric methods such as the iodination of protein have become the methods of choice mainly because of their high sensitivity; as little as 1ng of protein adsorbed onto 1cm<sup>2</sup> of material can be detected (Lee *et al.*, 1974; Chuang *et al.*, 1978).

This chapter reports an assessment of the blood compatibility of PHB and P(HB-HV) using radiolabeled albumin, fibrinogen and gammaglobulin. Adsorption isotherms have been determined and competitive adsorption has been investigated using binary protein solutions. The extent of platelet adsorption was also assessed using a <sup>51</sup>Cr radiometric method. Two primary reference materials (as defined by the National Heart Lung and Blood Institute) low density polyethylene (PEE) and polydimethylsiloxane (PDS), were used to provide a comparative index in the determination of the blood compatibility of PHB and P(HB-HV). In general PDS tends to be good materials for blood-material interaction whereas PEE is recognised to be reactive to platelets and a poor material for blood-material interaction (Lelah, 1983).

## Results

All experiments were carried out with labeled protein solutions in which the free iodine did not exceed 5%. If free  $^{125}\text{I}$  was found to be greater than 5% the solution was discarded as free iodine was found to preferentially adsorb onto the polymer surfaces thereby invalidating the data. The usual value of free  $^{125}\text{I}$  ranged between 2-5%. The percentage efficiency of the radioiodination process ranged between 60-70% and the amount of protein recovered from the column ranged between 96-98% according to the Lowry assay.

### 5.2. Preferential adsorption tests.

The results of preferential adsorption of the three radiolabeled proteins onto PHB and P(HB-HV) film surfaces are shown in table 5.1. At low ratios of labeled protein to unlabeled protein, the amount of protein adsorbed onto the polymer surfaces appeared constant for each of the three plasma proteins. However at high ratios of labeled to unlabeled protein (i.e. >50%) the labeled protein appeared to be preferentially adsorbed to the polymer surfaces. PEE and PDS were also seen to preferentially adsorb the labeled protein at a ratio >50% of labeled : unlabeled protein. This suggested that some conformational change in protein structure had occurred during radiolabeling, which enabled protein adsorption to occur to a greater extent.

### 5.3. Adsorption isotherms.

Figures 5.2-5.4 represent the adsorption isotherms obtained for albumin, fibrinogen and gammaglobulin onto each of the polymer surfaces. The data is summarized in table 5.2 where the slopes and

Table 5.1 Preferential adsorption of the radiolabeled proteins to PHB and P(HB-HV).

<sup>125</sup> I Protein %	PHB								
	Gammaglobulin			Fibrinogen			Albumin		
	1	2	3	1	2	3	1	2	3
1	0.16	0.32	0.32	0.11	0.16	0.10	0.26	0.22	0.19
5	0.27	0.26	0.18	0.13	0.17	0.11	0.21	0.25	0.25
10	0.24	0.26	0.24	0.16	0.14	0.14	0.24	0.27	0.24
20	0.19	0.58	0.30	0.23	0.18	0.17	0.27	0.30	0.31
50	0.53	0.48	0.37	0.20	0.26	0.25	0.64	0.58	0.55
100	0.92	0.91	1.16	0.36	0.39	0.31	0.84	1.10	0.94
	P(HB-HV)								
	Gammaglobulin			Fibrinogen			Albumin		
	1	2	3	1	2	3	1	2	3
1	0.25	0.29	0.25	0.10	0.15	0.12	0.19	0.16	0.15
5	0.25	0.21	0.31	0.18	0.16	0.16	0.12	0.21	0.18
10	0.22	0.33	0.20	0.12	0.15	0.17	0.17	0.15	0.16
20	0.37	0.36	0.30	0.21	0.13	0.18	0.24	0.21	0.26
50	0.38	0.34	0.36	0.20	0.19	0.16	0.23	0.31	0.27
100	1.20	0.45	1.03	0.22	0.27	0.27	0.59	0.64	0.66

Figures represent amount of labeled protein adsorbed to the each polymer surface in ug/cm<sup>2</sup>.

Total concentration of protein in solution in each case is 2mg/ml.

Figures represent the results of 3 individual experiments.

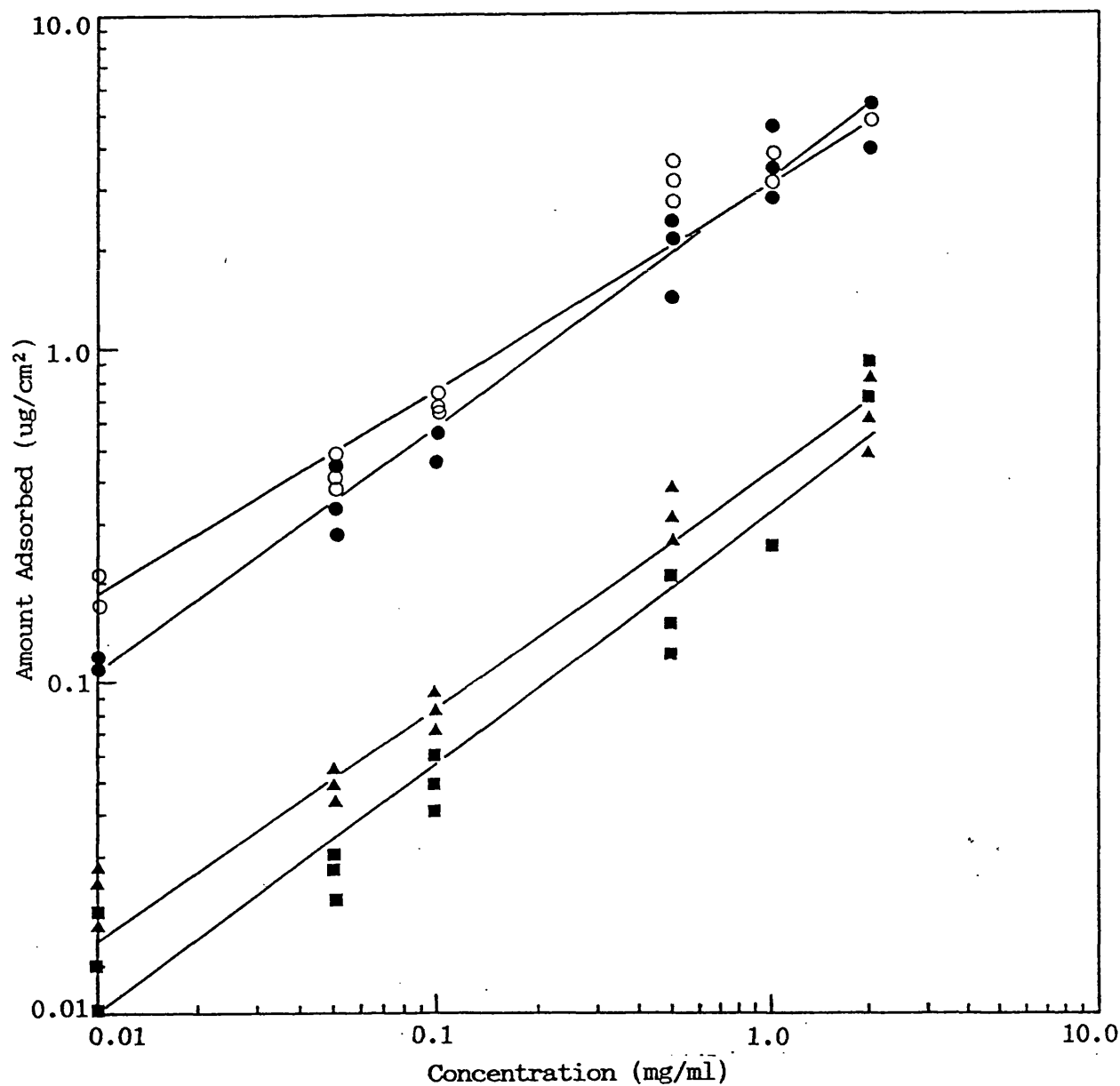


Figure 5.2. Adsorption isotherm for  $\beta$ -globulin onto PHB (●), P(HB-HV) (○), PDS (▲) and PEE (■). The data were obtained from films adsorbed for 2 hours at 37°C following thorough rinsing. Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.

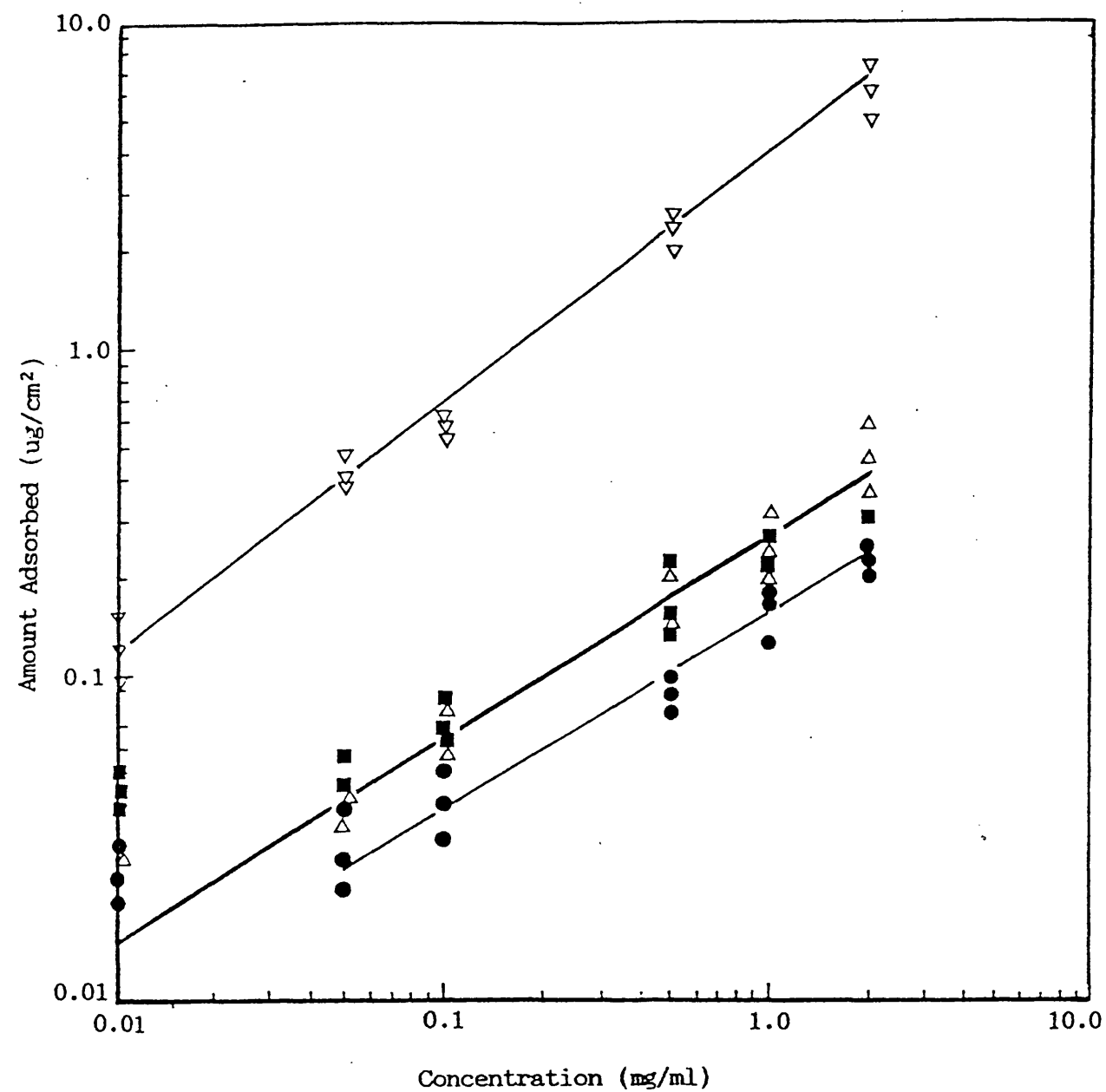


Figure 5.4. Adsorption isotherm for albumin onto PHB (●), PDS (Δ) PEE (■) and P(HB-HV) (▽). The data were obtained from films adsorbed for 2 hours at 37°C following thorough rinsing. Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.

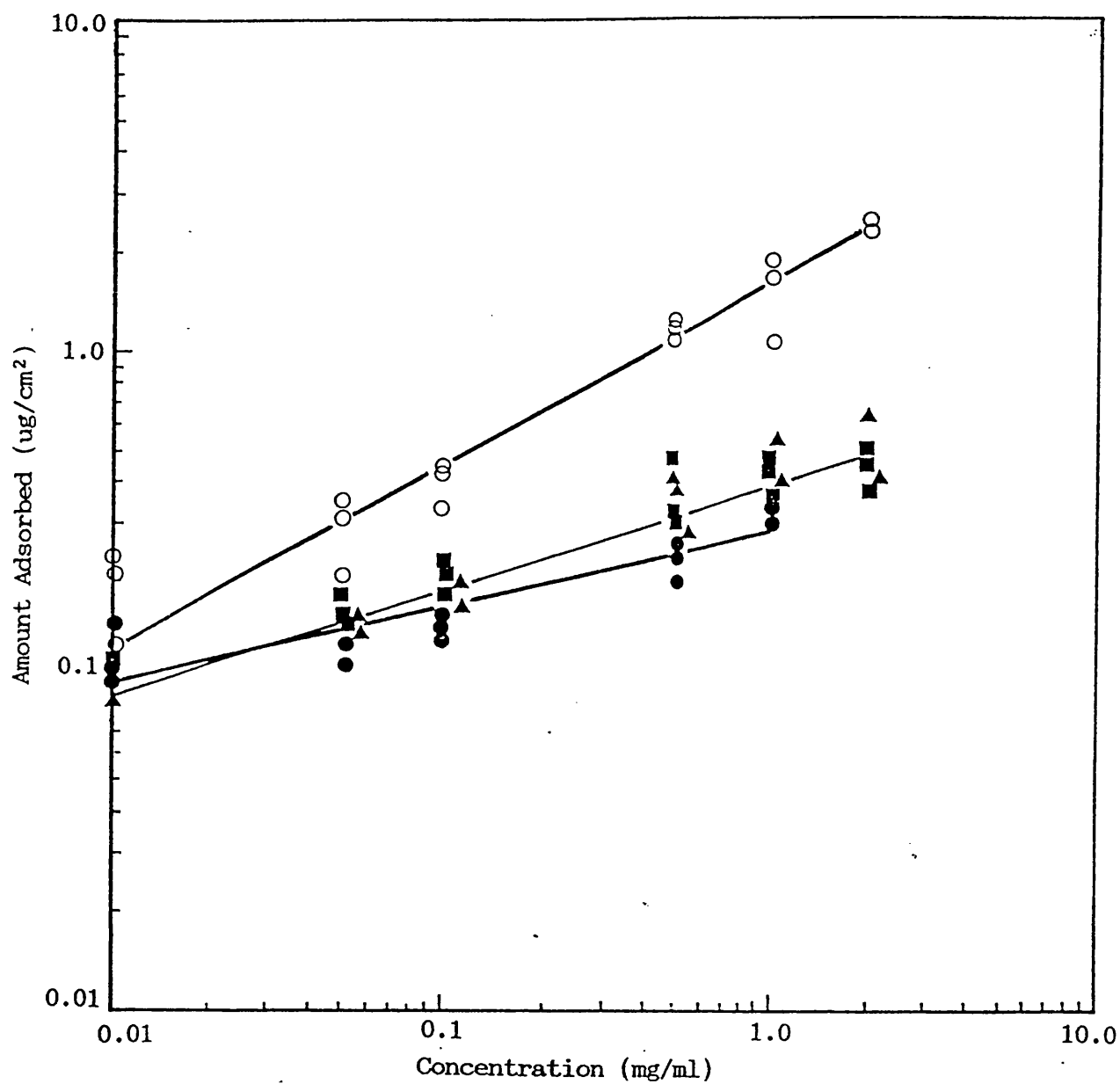


Figure 5.3. Adsorption isotherm for fibrinogen onto PHB (●), P(HB-HV) (○), PDS (Δ) and PEE (■). The data were obtained from films adsorbed for 2 hours at 37°C following thorough rinsing. Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.



### 5.2. Freundlich analysis of the plasma protein adsorption to polymers.

Protein		Slope		Intercept	
		b	$\bar{b}$	K	$\bar{K}$
PHB	Albumin	0.59	(0.01)	-0.16	(0.03)
	Fibrinogen	0.25	(0.02)	-0.60	(0.02)
	globulin	0.68	(0.02)	-0.05	(0.01)
P(HB-HV)	Albumin	0.73	(0.03)	-0.03	(0.01)
	Fibrinogen	0.53	(0.01)	-0.12	(0.02)
	globulin	0.68	(0.01)	-0.06	(0.02)
PEE	Albumin	0.68	(0.02)	-0.25	(0.05)
	Fibrinogen	0.29	(0.02)	-0.43	(0.13)
	globulin	0.70	(0.04)	-0.06	(0.03)
PDS	Albumin	0.69	(0.01)	-0.10	(0.01)
	Fibrinogen	0.29	(0.01)	-0.46	(0.07)
	globulin	0.67	(0.03)	-0.06	(0.01)

n = 3

Analysis of the isotherms was done according to the Freundlich isotherm:  $\log A = b \log C + K$ , where A is the adsorption ( $\mu\text{g}/\text{cm}^2$ ) and C is the bulk protein concentration ( $\text{mg}/\text{ml}$ ). b and K are the slope and the intercept of the line through the  $\log A$  vs  $\log C$  data.

intercepts for the regression lines which best fit the data are documented. Each adsorption isotherm showed that the protein adsorption was characteristic of a Freundlich plot over the concentration range studied. No saturation of protein adsorption was seen at high concentrations, and therefore the adsorption profile was unlike a Langmuir adsorption isotherm. However it is possible that at very high protein concentrations saturation would have been reached. Langmuir analysis may then have been appropriate.

Analysis of the Freundlich isotherm can be used to measure the strength or affinity of the adsorbate for the surface by calculation of the slope. Comparison of the slope values allow one to speculate which protein is most likely to be adsorbed when the polymer is exposed to whole blood or plasma. The results of the adsorption isotherm show that the rank order of affinities of the three proteins for each polymer is as follows;

PHB: gammaglobulin > albumin >> fibrinogen

P(HB-HV): albumin = gammaglobulin > fibrinogen

PEE: albumin = gammaglobulin >> fibrinogen

PDS: albumin > gammaglobulin >> fibrinogen

#### 5.4. Competitive adsorption.

Figures 5.5-5.7 show representative competition curves for various competing protein pairs. The concentration of unlabeled protein (I) which caused a reduction to 50% of maximal labeled protein (II) adsorption provides a convenient summary of the entire set of data. It is at this concentration that the two proteins compete equally for the adsorption sites, therefore if the two proteins were equally effective in competing for these sites, the 50% reduction point would

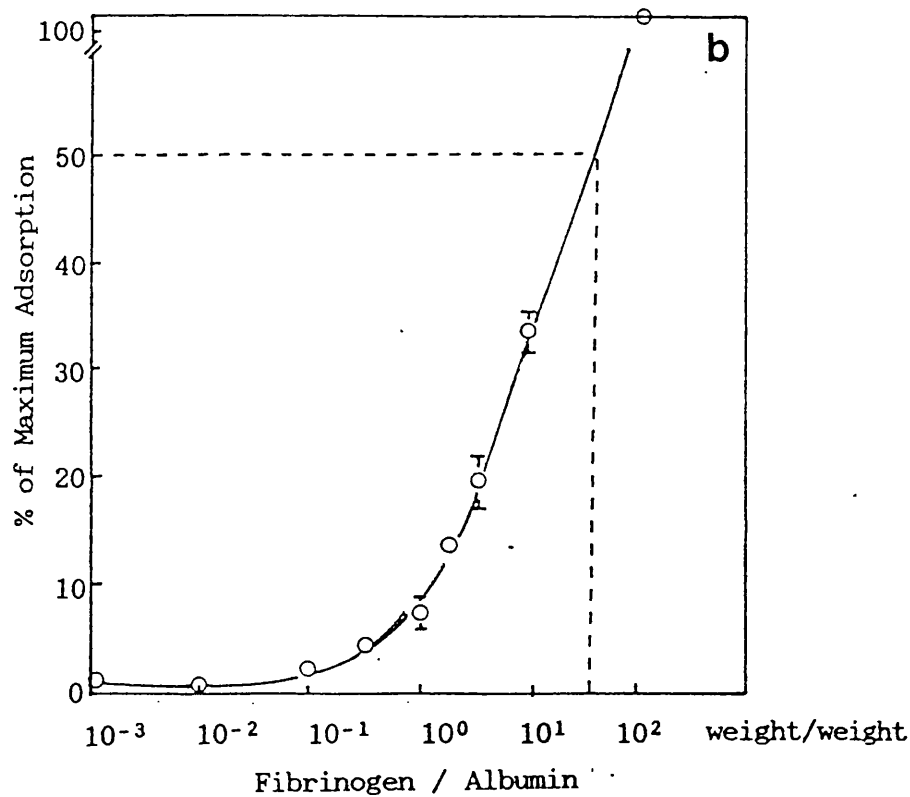
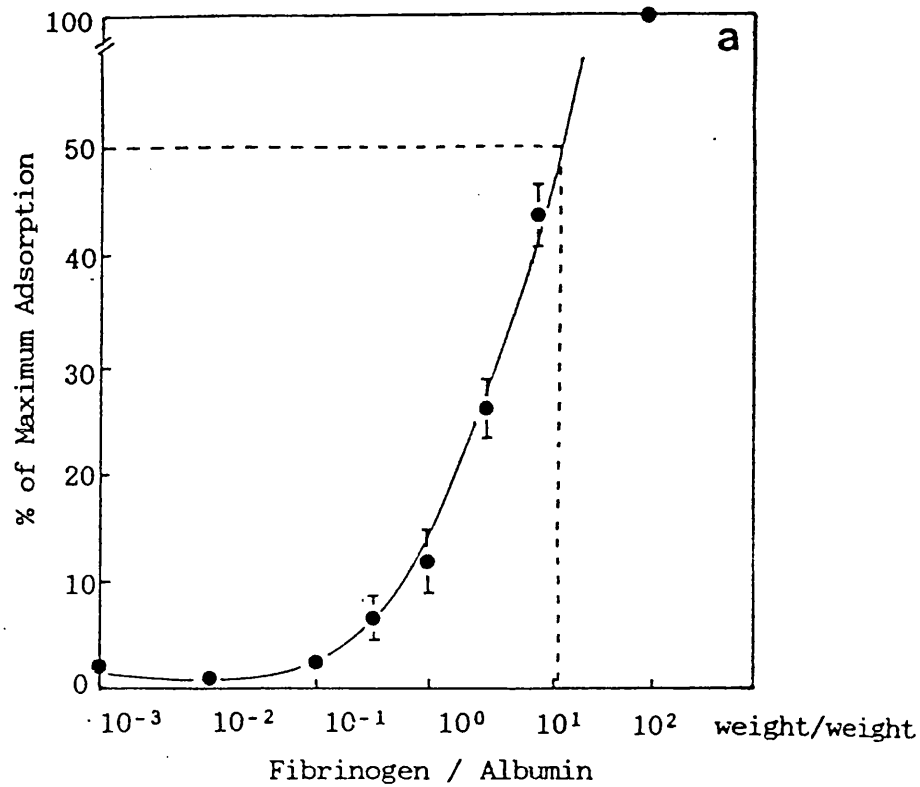


Figure 5.5. Competitive adsorption of plasma proteins to P(HB-HV). The percent of maximum adsorption of  $^{125}\text{I}$ -fibrinogen in the presence of decreasing amounts of albumin is plotted. The ratio of fibrinogen/albumin at which 50% maximal adsorption of labelled protein occurs is derived from this plot. (a) and (b) represent the results presented on Table 5.3, of experiments 2 and 3, respectively. Each point represents the mean of 3 experiments.

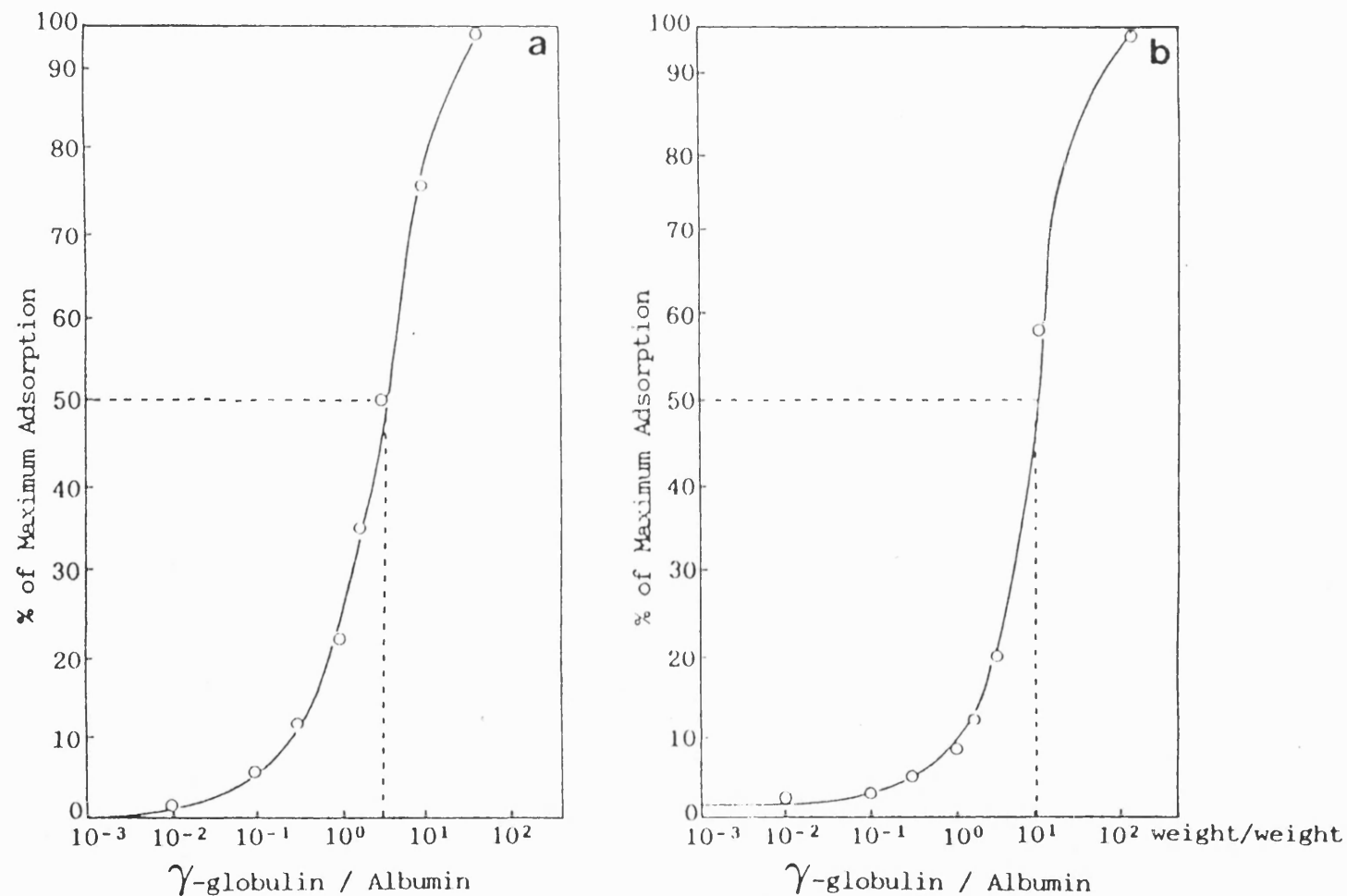


Figure 5.6. Competitive adsorption of plasma proteins onto P(HB-HV). The percent of maximum adsorption of  $^{125}\text{I}$ - $\gamma$ -globulin in the presence of decreasing amounts of albumin is plotted. The ratio of  $\gamma$ -globulin/albumin at which 50% maximum adsorption of labelled protein occurs is derived from this plot. (a) and (b) represent the results presented on Table 5.3 of experiments 1 and 2, respectively. Each point represents the mean of 3 experiments. The s.d. in each case was < 5% of the mean.

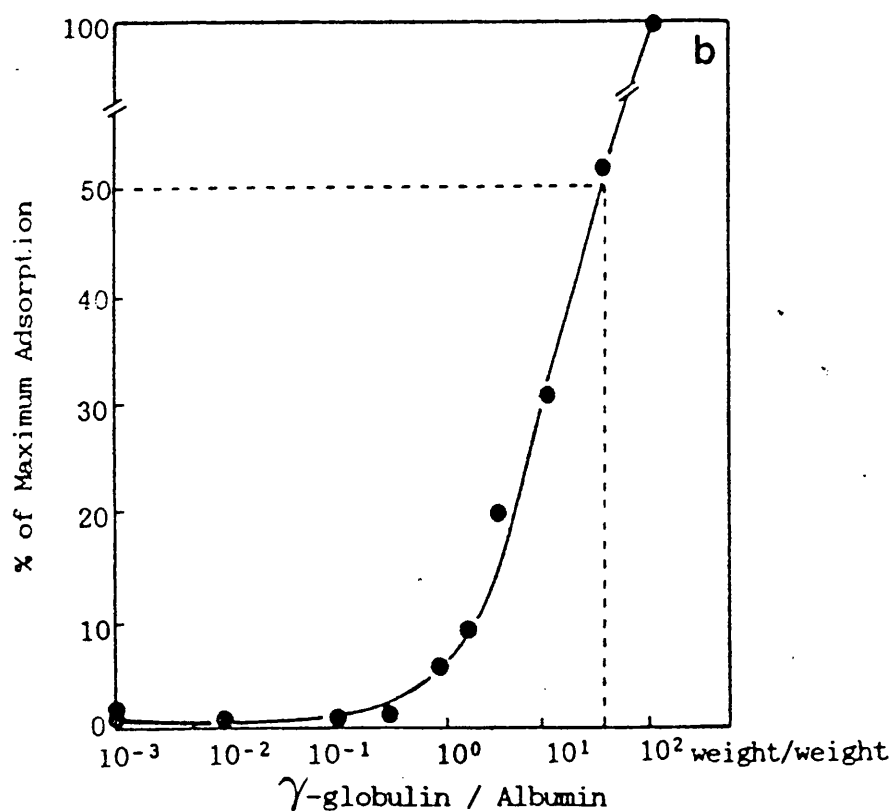
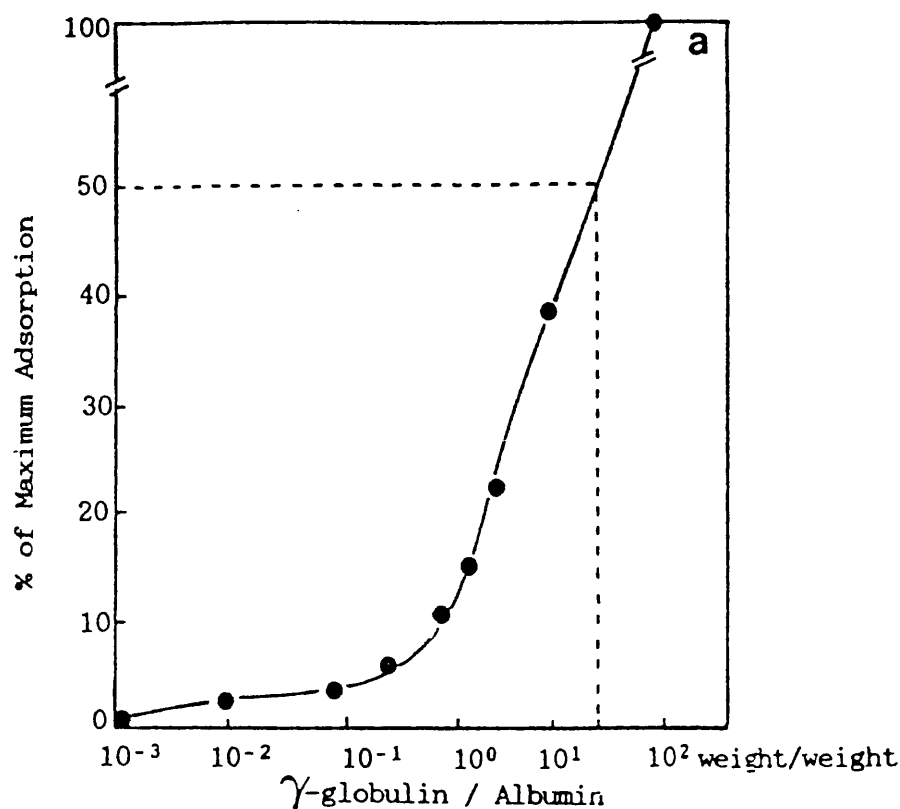


Figure 5.7. Competitive adsorption of plasma proteins onto PHB. The percent of maximum adsorption of  $^{125}\text{I}$ - $\beta$ -globulin in the presence of decreasing amounts of albumin is plotted. The ratio of  $\beta$ -globulin/albumin at which 50% of maximum adsorption occurs is derived from this plot. (a) and (b) represent the results presented on Table 5.3 of experiments 2 and 3, respectively. Each point represents the mean of 3 experiments. The s.d. in each case was  $< 5\%$  of the mean.

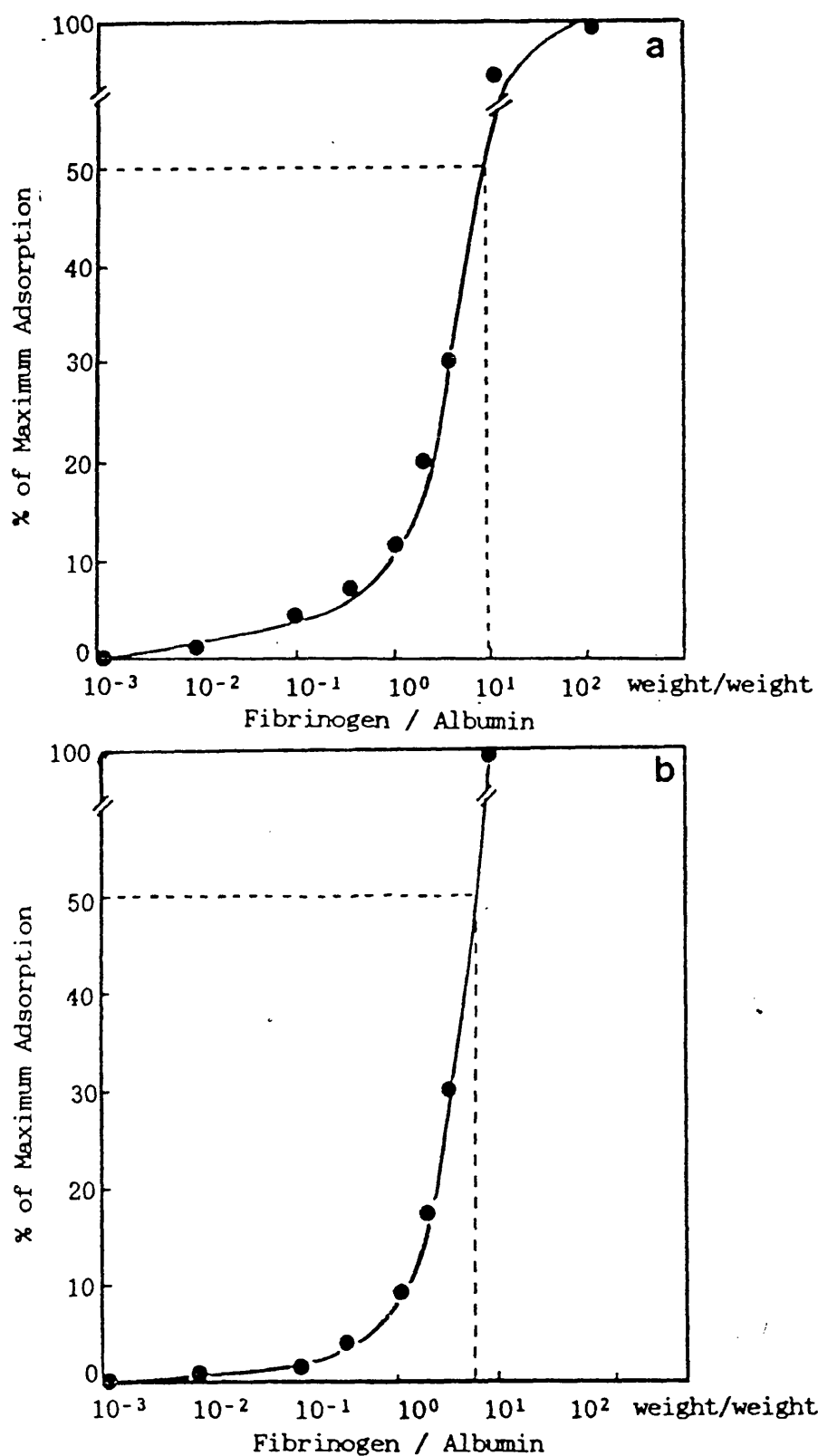


Figure 5.8. Competitive adsorption of plasma proteins onto PHB. The percent of maximum adsorption of  $^{125}\text{I}$ -fibrinogen in the presence of decreasing amounts of albumin is plotted. The ratio of fibrinogen/albumin at which 50% maximal adsorption of labelled protein occurs is derived from this plot. (a) and (b) represent the results presented on Table 5.3 of experiments 1 and 2, respectively. Each point represents the mean of 3 experiments. The s.d. in each case was  $< 5\%$  of the mean.

Polymer	Experiment no.	BF : BSA	BG : BSA	BG : BF
PHB	1	5 : 95	60 : 40	48 : 52
	2	3 : 97	50 : 50	40 : 60
	3	8 : 92	35 : 65	38 : 62
P(HB-HV)	1	75 : 25	5 : 95	36 : 64
	2	80 : 20	10 : 90	30 : 70
	3	60 : 40	20 : 80	35 : 65
PEE	1	10 : 90	70 : 30	95 : 5
	2	15 : 85	65 : 35	97 : 3
	3	10 : 90	75 : 25	95 : 5
PDS	1	10 : 90	60 : 40	90 : 10
	2	15 : 85	50 : 50	95 : 5
	3	7 : 93	45 : 55	92 : 8

Table 5.3 The ratio of labeled protein : unlabeled protein at which a reduction to 50% maximal adsorption of labeled protein occurred in binary competitive adsorption experiments.

BF = bovine plasma fibrinogen

BSA = bovine serum albumin

BG = bovine gamma globulin

occur at equal concentrations of the two proteins. If the 50% reduction point is reached at a lower concentration of the competing protein (I) than the labeled protein (II), the competing protein is considered to have a greater affinity for the polymer surface than the labeled protein.

The information obtained from the competition curves for each polymer is summarised in table 5.3. The concentration ratio of labeled (II) to nonlabeled protein (I) which causes a 50% reduction of maximal labeled protein (II) adsorption is stated. Fibrinogen adsorption to PHB surfaces was reduced to 50% of maximum adsorption by albumin at a protein ratio of fibrinogen to albumin 5 : 95, this implied that affinity of fibrinogen for PHB was 19 times greater than that of albumin. Gammaglobulin adsorption was reduced to 50% of its maximum by albumin at a mass ratio 48 : 52, which indicated that the affinities of gammaglobulin and albumin to PHB were approximately equal. Similarly gammaglobulin adsorption was reduced to 50% of its maximum by equal concentrations of fibrinogen. This competitive adsorption data for PHB indicated that fibrinogen equally adsorbs gammaglobulin when in competition with each other. The extent of protein adsorption as determined by the adsorption isotherms determines which plasma protein will be preferentially adsorbed and therefore whether PHB is likely to be thrombogenic. P(HB-HV) adsorbed 2.57 times more albumin than fibrinogen, 7.3 times more gammaglobulin than albumin and 1.94 times more gammaglobulin than fibrinogen. The competitive adsorption profile for P(HB-HV) was therefore, gammaglobulin > albumin > fibrinogen.

The primary reference material PEE adsorbed 7.3 times more fibrinogen than albumin, 2.33 times more albumin than gammaglobulin and 24 times more fibrinogen than gammaglobulin, therefore is



adsorption sequence is likely to be fibrinogen > albumin > gammaglobulin. PDS however adsorbed at equal concentrations of gammaglobulin and albumin, but 7.4 times and 11.5 times more fibrinogen than albumin or gammaglobulin respectively.

#### 5.4. Platelet adsorption:

The kinetic adhesion of platelets to the four biomaterial surfaces is shown in figure 5.8. For all polymers maximum platelet adhesion occurred within 10 minutes and thereafter a plateau effect was seen.

Table 5.4 illustrates the number of platelets which adsorbed from PRP onto untreated polymer surfaces and surfaces coated with a single plasma protein. As expected coating polymers with albumin prior to the adhesion experiments brought about a reduction in the number of platelets that adhered to the surface. PHB coated with fibrinogen did not incur an increase in the number of adsorbed platelets when compared to the uncoated polymer surface. However when PHB was coated with gammaglobulin a greater number of platelets adhered to its surface.

Platelet adhesion to PEE and P(HB-HV) was unaffected by coating with gammaglobulin or fibrinogen. PDS however did show an increase in platelet adhesion when coated with fibrinogen, although coating with gammaglobulin appeared to have no effect. Platelet adsorption to P(HB-HV) was considerably greater than to other polymers both in untreated polymer and protein-coated polymer experiments. These results are discussed in the following section.

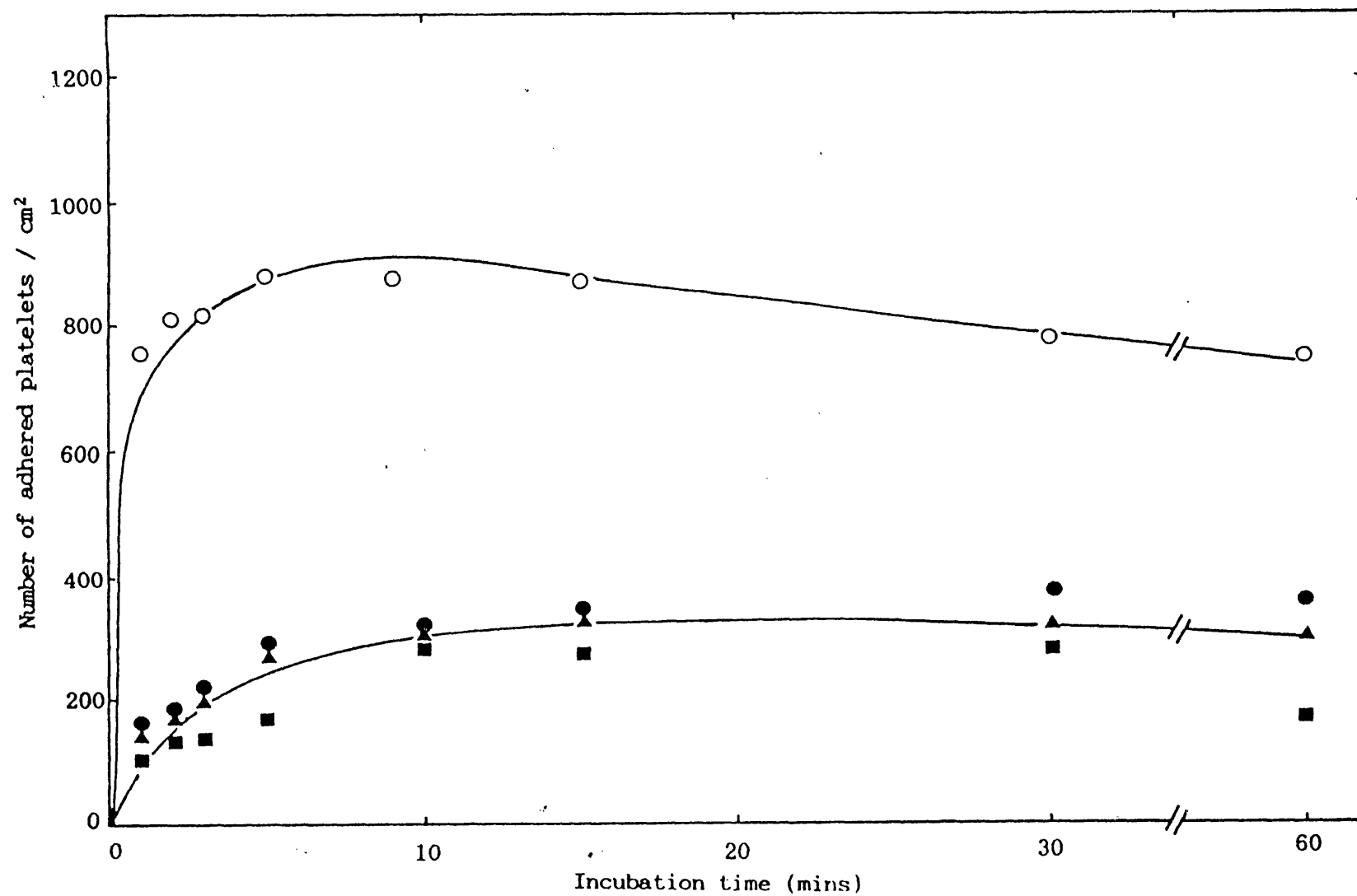


Figure 5.9. Platelet adhesion rate onto P(HB-HV) (O), PHB (●), PDS (▲) and PEE (■). Incubations were carried out at 37°C and the degree of adhesion assessed by  $\beta$ -counting. Each point represents the mean of 9 experiments. The s.d. in each case was < 5% of the mean.

Table 5.4 : Adhesion of platelets from PRP onto single protein coated or uncoated polymers surfaces.

Polymer	No. of adhered platelets/ $10^4\mu\text{m}^2$ mean (S.D)			
	Uncoated	BSA	BG	BF
PHB	148.9 (22.0)	160.3 (16.6)	434.5 (114.7)	155.6 (25.5)
	192.2 (32.5)	197.8 (45.8)	270.9 (68.8)	207.3 (34.7)
	275.6 (75.2)	158.3 (33.7)	389.5 (70.4)	239.1 (59.8)
P(HB-HV)	700.1 (48.1)	438.1 (28.2)	717.4 (51.3)	680.5 (40.9)
	742.6 (80.8)	312.8 (95.6)	774.9 (25.9)	736.1 (56.4)
	778.6 (54.2)	363.6 (32.9)	789.9 (14.8)	750.6 (38.9)
PEE	323.7 (20.2)	212.1 (30.8)	235.8 (37.3)	261.9 (48.1)
	272.6 (69.1)	248.5 (17.9)	291.4 (25.6)	287.1 (36.2)
	340.9 (38.4)	221.8 (50.2)	306.9 (50.6)	299.0 (18.7)
PDS	320.5 (16.9)	224.4 (23.1)	307.1 (63.9)	375.8 (24.1)
	310.4 ( 7.4)	217.5 (60.4)	363.8 (21.0)	427.2 (39.4)
	289.2 (32.6)	204.3 (36.1)	324.2 (42.6)	406.8 (18.7)

n = 9 in each of the three separate experiments.

ANOVA test performed on the three separate experiments in each test group showed no significant variation between experiments.

#### 5.4 Discussion

Investigations into the thrombogenicity of biomaterials have shown that the composition of the surface protein layer adsorbed onto biomaterials, will greatly affect its thrombogenic character (Young *et al.*, 1982). This adsorbed protein layer will affect the adherence of platelets and their subsequent activation (Adams and Feuerstein, 1981; Young *et al.*, 1982). Much research has been directed towards establishing the pattern of adsorption of three plasma proteins, albumin, gammaglobulin and fibrinogen and their modification of surface properties and therefore their subsequent influence on cell surface interactions. In more recent years trace plasma proteins, such as fibronectin and von Willebrand factor, in addition to the main plasma proteins have been studied to establish their role in thrombogenicity (Vroman *et al.*, 1980 and Kochwa *et al.*, 1977). It is generally accepted that on natural surfaces, plasma proteins remain in their natural state and are reversibly adsorbed (Iorclanskii and Zaikov, 1983). Conversely, plasma proteins which adsorb and accumulate onto polymer surfaces may exhibit changes in their conformation and orientation, and subsequent thrombotic events such as platelet adhesion may be influenced greatly by the nature of this protein layer (Morrissey, 1977; Packman, 1969 and Baier, 1977).

An important consideration in such studies is the effect of radiolabeling the protein on its affinity for a polymer surface. In this study the results of preferential adsorption studies indicated that denaturation of labeled protein had an effect on adsorption at high mole ratios of labeled protein. This supports the findings of Grant *et al.* (1977). However, Bornzin and Miller, (1982) found that the measured adsorption of albumin and fibrinogen on two different

surfaces was independent of the mole fraction of labeled protein, indicating no effect of radiolabeling.

Adsorption isotherm data suggested that P(HB-HV) had a significantly greater ( $p < 0.01$ ) affinity for fibrinogen proteins when compared to the primary reference materials and its homopolymer and a significantly greater ( $p < 0.01$ ) amount of the protein was adsorbed to its surface at any one concentration when compared to the other polymers. PHB, P(HB-HV), PEE and PDS had equal affinity for gammaglobulin. All polymers as indicated by the slope value appeared to have similar affinities for albumin, again P(HB-HV) adsorbing significantly more ( $p < 0.01$ ) when compared to the other polymers over the concentration range used. Protein adsorption affinities for each of the polymer surfaces can be summarised as gammaglobulin = albumin > fibrinogen as indicated by the slope values for each polymer surface. The affinity of the polymer surfaces for gammaglobulin implied that all polymers could be potentially thrombogenic. However, McCoy *et al.*, (1989) reported that PEE and PDS were relatively benign in their interaction with blood and could be assumed nonthrombogenic. The degree of thrombogenicity must therefore be determined by factors other than the adsorption affinity from the single protein solutions.

Hanson *et al.*, (1986) suggested that it was the amount of plasma protein irreversibly adsorbed to the polymer surface, and its competition with other plasma proteins which determined the pattern of plasma protein adsorption and therefore subsequent adhesion and activation of platelets.

The competition adsorption data for the polymers from binary protein solutions show the two primary reference materials adsorbed in the pattern of fibrinogen > albumin > gammaglobulin, and as seen

from the adsorption isotherm data the amount adsorbed in terms of concentration was greatest for fibrinogen > albumin > gammaglobulin. These results indicate that platelets are likely to adsorb to the polymer-protein surfaces when in contact with whole blood, but the extent of which can only be determined by platelet adhesion experiments. PHB appeared to adsorb gammaglobulin and albumin to the same extent when in competition in a binary solution. Gammaglobulin was adsorbed to a slightly greater, but insignificant ( $p>0.05$ ), extent when in competition with fibrinogen in binary solution. However fibrinogen was significantly adsorbed ( $P<0.01$ ) to PHB when in competition with albumin. These results suggest that when all three plasma proteins are in solution then gammaglobulin and fibrinogen would preferentially adsorb. P(HB-HV) competitively adsorbed the proteins in the order gammaglobulin > fibrinogen > albumin. The overall plasma protein adsorption profile indicates that platelets are likely to adhere to all polymer surfaces when in contact with blood but the extent of which can only be determined by platelet adhesion experiments.

It is agreed by most investigators in the field of blood compatibility that adsorbed fibrinogen and gammaglobulin facilitate and enhance platelet adsorption, whereas albumin behaves as a passivating agent (Kim *et al.*, 1977 and Mosher, 1981). This can be explained by the following platelet adhesion mechanism: following protein adsorption platelets approach the proteinated surface and react with glycoprotein receptors to form a complex which is catalysed by a glycosyl transferase enzyme (Bosmann, 1971, 1972 and Lee *et al.*, 1974, 1979). The stability of the complex determines the strength and duration of the reaction and therefore the formation of a thrombus. Only fibrinogen and gammaglobulins contain these

glycoprotein receptors and are therefore capable of forming a platelet protein complex. Albumin does not contain any glycoprotein receptors and therefore does not have the capacity to form such complexes, which may be the reason for its nonthrombogenicity.

The results of the platelet adhesion experiments onto protein coated polymers agreed with the above hypothesis in that all polymers precoated with albumin decreased the number of platelets adhering to the polymer surface. However fibrinogen and gammaglobulin coated polymer surfaces appeared to adsorb platelets to the same extent as the noncoated polymer ( $p > 0.05$ ), except for gammaglobulin coated PHB surface when adsorption was significantly greater ( $p < 0.01$ ) than that for the uncoated polymer. The adsorption of platelets therefore also appears to be due to a surface property of the polymer. Platelet adhesion to coated or uncoated P(HB-HV) was greater ( $p < 0.01$ ) than adhesion to all other polymers. Whereas uncoated and albumin or fibrinogen coated PHB adhered the least number of platelets when compared to the other polymers. Where gammaglobulin coated PHB showed an increase in platelet adherence numbers this was comparable to the uncoated primary reference materials, indicating good blood compatibility of this polymer.

In summary, the results of both plasma protein and platelet adsorption to the polymer surfaces *in vitro*, gave a thrombogenicity index of  $P(HB-HV) > PEE > PDS > PHB$ . The relation of PEE to PDS in this index agreed with other research carried out on these reference materials (Neumann *et al.*, 1980, 1983; Lelah *et al.*, 1983; Mason *et al.*, 1983 and Pelzer and Heimberger., 1986). McCoy *et al.*, (1989) also showed PDS to be less thrombogenic than PEE *in vitro* and *ex vivo*, however PDS in long term experimentation adhered a greater number of platelets and therefore the formation of a mural thrombus



increased. Hanson *et al.*, (1980) showed little difference in the blood compatibility of the two primary reference materials and therefore both were pronounced blood compatible. However, Lelah *et al.*, (1983) and Mohammed *et al.*, (1976) showed significant platelet reactivity and adsorption on PEE. Contradictory literature reports emphasises the need for other primary reference materials to be used in blood compatibility assessment so that interlaboratory comparisons and compatibility index can be made. The similarity in surface properties and blood compatibility of these reference materials and their commercial counterparts indicates the need for less thromboresistant as well as more polar reference materials. However, as many extracorporeal devices and pumps are made from PDS (Silastic) tubing (Neumann *et al.*, 1983; Lelah *et al.*, 1983) any material with a lower thrombogenicity index to this material can be considered to have good blood compatibility.

The question arises, why do certain polymer surfaces preferentially adsorb albumin and are therefore nonthrombogenic while others adsorb fibrinogen or gammaglobulin and are potentially thrombogenic. Many investigators believe that surface hydrophobicity/hydrophilicity plays an important part in plasma protein adsorption and subsequent platelet adherence. Lyman *et al.* (1965) established a relationship between surface free energy of uncharged, hydrophobic polymers and coagulation time of the plasma or blood in contact with them. The more hydrophobic the polymer the less thrombogenic it appeared. Therefore in theory a prediction of the thrombogenicity of a material could be predicted by its surface wettability i.e. hydrophobicity (Absolom *et al.*, 1987; Steinberg *et al.*, 1989). The greater the value of contact angle the greater the hydrophobicity of the material.

Table 5.5 lists contact angle data for P(HB-HV) and PHB, and the primary reference materials. The data showed that the sequence of hydrophobicity of these polymers was:

$$\text{PDS} > \text{PEE} > \text{PHB} > \text{P(HB-HV)}$$

Of the polymers studied, P(HB-HV) had the lowest contact angle and therefore appeared to be the most hydrophilic material studied. Hydrophilicity has been reported to equate with potential thrombogenicity, (Baier, 1984) this was borne out in the experiments where high fibrinogen and gammaglobulin adsorption and the greatest platelet adhesion occurred to P(HB-HV). The fact that the uncoated polymer surface adhered platelets equally as well as the surfaces coated with gammaglobulin and fibrinogen suggested a different mechanism of adherence was involved. A second hypothesis for control of platelet adhesion has been reviewed by Neumann *et al*, (1983). The minimum interfacial free energy theory states that as interfacial free energy (solid/water) approaches zero, the driving force for protein and platelet adsorption decreases (Coleman *et al*, 1982). Surfaces with low interfacial free energy are said to be hydrophobic and those with high interfacial energy are hydrophilic. This theory reasons that hydrophobic surfaces present less driving force for spreading and attachment. In agreement with this, surfaces with lower interfacial free energy adsorb fewer platelets than the more hydrophilic surfaces (Lindsay *et al*, 1980; Neumann *et al*, 1978, 1980.)

Brash (1969) reported that the most important general binding mechanism of protein to polymer surface, is that of hydrophobic interaction, i.e. the interaction of nonpolar groups in aqueous media. Proteins are known to contain hydrophobic domains in their surface which would be available to interact with hydrophobic contacting surfaces, typified by PDS, PEE and PHB. The degree to

Table 5.5. Contact angle data for polymers: Air/water from sessile drop measurement.

<u>Surface</u>	<u>Contact L</u>	<u>Ref.</u>
PRM-SR	90	McCoy <i>et al</i> (1989)
PRM-PE	89	McCoy <i>et al</i> (1989)
Silastic SR	80	McCoy <i>et al</i> (1989)
Intramedic PE	93	McCoy <i>et al</i> (1989)
PHB (380K)	85 (smooth) 83 (rough)	Akhtar (1989)
P(HB-HV) 12.6% mole hv Mw 760K	67 (smooth) 74 (rough)	Akhtar (1989)

which any particular protein is adsorbed to the polymer surface depends on whether the surface is hydrophilic or hydrophobic, charged or neutral and polar or apolar. Plasma proteins in general have been reported to have a greater affinity for hydrophobic surfaces than hydrophilic surfaces (Neumann *et al.*, 1983). Albumin is a protein which has hydrophobic domains used for transporting low molecular weight molecules in the blood; adsorbing albumin to the surface could expose the hydrophobic domains and would therefore increase the hydrophobicity of the surface. Since highly hydrophobic domains are thought to be blood compatible, an explanation of the protective effect of albumin coating may be due to exposure of its hydrophobic domains (Kotte-Marchant *et al.*, 1989). Pre-coating of polymer surfaces with albumin has been used to improve the blood compatibility of many prosthetic devices. Other methods to improve the blood compatibility of polymers include heparinisation (Chokalis and Sefton, 1989). However, contradictory evidence in the use of albumin pre-coating or heparinisation (Wilson, 1981; Ito *et al.*, 1986) has lead investigators to elaborate on the mechanisms of blood compatibility and therefore work on other methods to achieve better blood compatibility of polymers.

To date no synthetic material has been found to be entirely passive to blood. In 1978 Mansfield and Wechezak and Burkel *et al.* (1978) lined both synthetic and natural materials with cultured epithelial cells in an attempt to produce a nonthrobogenic blood compatible surface, however difficulty arose in developing a continous lining and the great length of time required to prepare the prosthesis. Over the past 25 years, much research has been directed towards finding biomaterials for use as blood contacting surfaces in cardiovascular devices, heart valves, large diameter vascular grafts

and in equipment for blood storage and in devices used in renal dialysis. Biodegradable materials are among newer concepts emerging in the development of biocompatible small caliber vascular prostheses, in that they could function as scaffolds for the regeneration of an intimated wall. Bowald *et al.*, (1980) studied the implantation of a double layer of absorbable polyglactin 910 mesh tubes in the descending aorta of young pigs, and found that within 40-60 days this had become completely replaced by a new endothelium. Lauritzen (1983) used polyglycolic acid as an absorbable vascular prosthesis in the femoral arteries and veins of rats. Four months after implantation 50% of the veins became patent, the polyglycolic acid hardly detectable after 4 weeks. Lei *et al.*, (1985 and 1986) tested microporous compliant biodegradable vascular grafts prepared from mixtures of polylactic acid and polyurethane in the abdominal aorta of rats. The *in vitro* studies on plasma protein and platelet adsorption to the biodegradable polymers PHB and P(HB-HV), has shown that PHB exhibits many characteristics indicative of a thromboresistant material. As PHB is also biodegradable there is great potential in the use of this material in vascular grafts.

#### 5.4. Conclusions.

1) There was preferential adsorption of radiolabeled protein at high labeled :unlabeled protein ratios to both polymer surfaces, indicating that some conformational change in protein structure had occurred during radiolabeling which enabled protein adsorption to occur to a greater extent.

2) Adsorption of plasma proteins from single protein solutions was characteristically of the Freundlich isotherm.

3) Competitive adsorption of proteins in binary solution showed that the primary reference materials adsorbed in the pattern, fibrinogen > albumin > globulin; PHB adsorbed proteins in the pattern, globulin = albumin > fibrinogen; P(HB-HV) adsorbed in the pattern globulin > fibrinogen > albumin.

4) P(HB-HV) adsorbed the greatest number of platelets, but PHB adsorbed platelets to the same extent as the primary reference materials.

5) Fibrinogen coated polymer surfaces adsorbed a greater number of platelets than the uncoated surface.

6) PHB, PEE and PDS have the potential of good blood compatibility, whereas P(HB-HV) showed signs of poor blood compatibility.

## CHAPTER 6. *In vivo* biocompatibility studies. Assessment of the soft tissue inflammatory response.

### 6.1 Introduction

Many techniques employed for the evaluation of biomaterials cover an assessment of the inflammatory reaction caused by the materials (Callis *et al.*, 1988; Marion *et al.*, 1980). The stimulus necessary to initiate an inflammatory response can vary greatly, but it is the continued presence or absence of the stimulus that dictates the inflammatory response. Attempts to neutralise or eliminate the inflammatory stimuli either by macrophage uptake or formation of a fibrous capsule around the stimuli can lead to eventual problems with the implanted material such as formation of necrotic lesions or tissue reaction (Coleman *et al.*, 1974).

Surgery involved in implantation or even simple injection may induce the initial reactions which include a series of interdependent events that begin with haemodynamic changes, (Joon Du Park, 1984) followed by alterations in the vascular permeability (Wilhem, 1977). The increased permeability of the adjacent vasculature promotes the transport of protein rich inflammatory fluid or exudate into the extravascular tissues and wound site (Meachim and Pedley, 1981). Simultaneously circulating leucocytes interact with the stimulatory factors, adhere to the blood vessels endothelia and pass through the vessel wall and into the vascular tissue around the implant.

The intensity and duration of the response is controlled by a variety of mediators, including histamine and prostaglandins, and determined by the size and nature of the implanted material (Rigdon, 1974; Stokes and Cobian, 1982; Turner *et al.*, 1973; Meachim *et al.*, 1982). The dynamic variation of the components of the inflammatory

and healing responses determine the biocompatibility of the material. A brief cellular response of low intensity is indicative of tissue compatibility (Marchant and Anderson, 1986). Interactions at the polymer interface is important particularly in relation to cellular function and organisation. The majority of studies on the effects of polymer implants on cellular organisation and function have been based on histological observations and morphological evaluations (Clarke *et al.*, 1974; Thomsen and Ericson, 1985; Williams and Blayney, 1987; Tamargo *et al.*, 1989). Using these techniques information on the post implantation changes occurring within specific cell populations can be gained, but little is gained regarding changes in cell function.

The acute phase of inflammation is characterised by the preferential migration of PMN's (the neutrophil), peak migration occurring within the first 72 hrs following injury (Dickinson and Hiltner, 1981). Macrophages and lymphocytes predominate at later stages of the inflammatory process (Salthouse *et al.*, 1973,1975). The cellular response evoked by implantation of a foreign object is accompanied by a variety of enzyme patterns. These are characteristic and indicative of the functional capacity of these cell populations adjacent to the implants and can therefore be used as markers in assessment of the inflammatory response and also the biocompatibility of implanted material. An enzyme histochemical approach to the assessment of biomaterial's tissue compatibility, founded by Salthouse and coworkers (1972, 1973 and 1976), is now an established technique.

The most important enzymes associated with the PMN's and macrophages of the inflammatory response are the lysosomal hydrolases, alkaline and acid phosphatase respectively. Alkaline



phosphatase is usually only observed at the implant site from 1-7 days in association with the PMN's. This enzyme is located in the specific granule of the PMNs and its release is through exocytosis resulting from activation (Salthouse and Williams, 1969). The level of activity of this enzyme will indicate the severity of the initial acute inflammatory response induced at the implant site by the implanted material. Acid phosphatase is the most prevalent hydrolase resulting from the presence of macrophages at implant sites. In general, but depending on the implant material, reactivity is noticeable at 4 days and reaches a maximum at ~14 days, activity then declines until fibrosis at the site is complete. With highly irritating polymer implants acid phosphatase activity is very greatly increased (Black, 1981). Thus acid phosphatase is particularly useful as an indicator of macrophage phagocytic activity at the site of implantation, the significance of which was discussed in chapter 4.

Raekallio (1970) showed the value of enzyme histochemistry for the assessment of biocompatibility of polymeric materials in wound healing studies and Salthouse and Williams (1969) used similar techniques to evaluate the tissue reaction to surgical sutures. Since then the use of enzyme histochemistry has continued to be used as an indicator of biocompatibility by a number of scientists (Salthouse and Willigan, 1972; Marchant *et al.*, 1983, 1984, 1987; Spilizewski *et al.*, 1987).

In this study three methods were used to assess the *in vivo* soft tissue inflammatory response of the rat to PHB, P(HB-HV) and their degradation products:

- 1) Histological studies to allow an overall assessment of the tissue reaction,
- 2) Changes in vascular permeability.

3) Changes in enzyme activity.

## 6.2. Results.

The uninjected, dry microspheres prepared as described in appendix 1, appeared uniformly spherical in shape when examined using light microscopy. When examined using scanning electron microscopy smaller particles were found adhering to the surface of each microsphere so that their surfaces appeared rough. The average diameter of the microspheres was 40  $\mu\text{m}$ . These were injected into the hind thigh of male Wistar rats and left 1-40 days when the muscle was examined.

### 6.2.1. Macroscopic observations.

Macroscopically, the injection sites of PHB and P(HB-HV) microspheres appeared as pockets of microspheres between muscle fibres at the proximal area of injection. There was a localised slight reddening in the muscle tissue surrounding these injection sites. No dependence on time of implantation could be observed by visual inspection. The injection sites following injection of the monomers also showed slight reddening in the muscle tissue, no suppuration or necrosis was evident.

Film implants were prepared as described in appendix 1 and inserted into rat thigh for 1-40 days. On inspection of the film implant sites for PHB and P(HB-HV), a mild acute inflammatory response characterized by reddening and oedema was seen during the first 10 days. At day 40 both films were encapsulated by a thin fibrous capsule, unaccompanied by chronic inflammation or necrosis. On removal of the capsule the films were seen to remain intact. The PMMA film evoked a similar response, however the fibrous capsule around PMMA film appeared to be of greater density and thickness.

### 6.2.2. Histological observations.

The sites of sham injections and vehicle injections (1%

methylcellulose in 0.9% saline) appeared to be "normal" histologically (see figs 6.1 and 6.2 respectively), i.e. cellular organisation proximal to the injection site did not differ to the cellular organisation seen in normal muscle tissue of the rat. On examination of the tissue proximal to the injection site of PHB microspheres, after 7 days a majority of normal cells with a few round cells and giant cells were evident, which were still apparent after 14 days (see figs 6.3 and 6.4 respectively). The site of monomer HB (100mg/ml) injection, after 7 and 14 days, appeared normal histologically.

Seven days following the injection of P(HB-HV) microspheres severe inflammation was evident as characterised by muscle fibrosis and abcess formation (see fig. 6.5). After 14 days this inflammatory response had resolved and was characterized by the presence of "foamy" cells and giant cells, (see fig. 6.6). The corresponding monomer HV (100mg/ml) 7 days after injection indicated a minor inflammatory response as shown by infiltration of a few PMN's into the injection site (see figure 6.7). 14 days after HV injection the tissue appeared histologically normal. The positive control, PAA, at both 7 and 14 days following its injection, showed a vast number of round cells in the vicinity of the injection site (see figures 6.8 and 6.9).

The above results indicated a different response to injection of PHB and P(HB-HV) microspheres. The histological examination of P(HB-HV) injection into rat muscle tissue was therefore repeated using 3 separate batches of injection on different occasions. In each case the histologist performed the assessment without knowledge of the identity of the formulation. The results were the same for each batch of P(HB-HV); this polymer therefore appeared to evoke a

Table 6.1 Histological examination of tissue after intramuscular injection of test materials.

Polymer mg/ml	7 days					14 days				
	PMN's	Macrophages	FBGC	Muscle Fibrosis	Necrosis	PMN's	Macrophages	FBGC	Muscle Fibrosis	Necrosis
PHB 10	-	+	++	-	-	-	++	++	-	-
100	-	+	++	-	-	-	++	++	-	-
P(HB-HV) 10	-	++	++	+	+	-	+	++	-	-
100	-	++	++	+++	+++	-	+	+++	-	-
HB 10	+	+	-	-	-	-	+	-	-	-
100	+	+	-	-	-	-	+	-	-	-
HV 10	+	++	-	-	-	-	+	-	-	-
100	+	++	-	-	-	-	+	-	-	-
PAA 100	-	++	+++	+	+	-	+	+	-	-
Zymosan 100	+	+++	++	+	++	-	+++	+	+	++
µg/ml										
Vehicle	+	+	-	-	-	-	+	-	-	-
Sham	+	+	-	-	-	-	+	-	-	-

+++ severe response

++ moderate

+ mild

Vehicle = 1% methylcellulose in 0.9% saline.

Observations are made on histological sections taken from 4 rats with each treatment.

more severe inflammatory response than PHB. The results of the histology assessment are summarized in table 6.1.

### 6.2.3. Effect on vascular permeability

Changes in vascular permeability were assessed by the extent of extravasation of Evans Blue in the vicinity of the site of injection. The dye was assayed by spectrophotometry at 612nm. Table 6.2 shows the amount of Evans Blue recovered from the injection site after administration of various agents. Irritants (HCl and NaOH) produced highly significant increases ( $p < 0.001$ ) in the vascular permeability when compared to changes produced by injection of the control vehicle (1% methylcellulose in 0.9% saline). However intradermal injection of 5HT and histamine, known mediators of the inflammatory response, did not significantly increase the vascular permeability. Zymosan (100ul/ml) also resulted in highly significant increases ( $p < 0.001$ ) in vascular permeability, but PHB and P(HB-HV) caused no significant changes in vascular permeability over a 4 hour period. Both monomers, HB and HV, resulted in significant increases ( $p < 0.01$ ) in vascular permeability at 10mg/ml and 100mg/ml, however sucrose injection (500mg/ml) also significantly increased vascular permeability suggesting that osmotic effects were responsible. The effect of sham injections on the vascular permeability was lower than all other injections including vehicle injection suggesting that injection of 0.1ml vehicle has an effect and invokes its own inflammatory response.

Table 6.2 Degree of extravasation as a consequence of intradermal administration of various mediators.

Evans Blue (ug) recovered in the skin after i.d. injection (0.1ml)

		Hrs after injection	
		2	4
Mediator (M; pH )			
NaOH	(0.1M; 12.84)	** 74.20 (2.06)	** 79.10 (7.21)
HCl	(1.0M; 0.45 )	** 96.90 (2.89)	** 74.00 (5.33)
Histamine	(1mM ; 9.37 )	31.40 (16.22)	14.80 (4.21)
5-HT	(0.1mM; 6.32)	13.60 (4.95)	14.00 (2.89)
HB	(10mg/ml; 7.61)	* 26.40 (4.23)	* 35.40 (4.95)
HB	(100mg/ml; 7.82)	* 40.60 (9.26)	* 38.80 (2.81)
HV	(10mg/ml; 7.24)	* 36.30 (4.95)	* 24.00 (2.89)
HV	(100mg/ml; 7.18)	** 61.10 (4.38)	* 41.50 (7.21)
PHB	(20mg/ml; 7.26)	9.10 (4.57)	10.40 (1.20)
PHB/HV	(20mg/ml; 7.30)	13.00 (1.00)	12.20 (4.95)
Polymer vehicle	(7.36)	11.80 (1.59)	12.20 (1.94)
Sucrose	(500mg/ml; 7.46)	14.60 (1.59)	12.30 (2.81)
Zymosan	(100ug/ml 7.35)	** 89.63 (6.60)	* 85.11 (3.55)
Sham		5.84 (2.58)	5.12 (1.75)

Polymer vehicle = 1% methylcellulose in 0.9% NaCl

Sham = penetration of tissue with needle without injection of fluid

Figures represent the mean (s.d.) of three experiments

\*\*Statistically different at the 99.9% confidence level ( $p < 0.001$ ) compared to the vehicle control.

\*Statistically different at the 99% confidence level ( $p < 0.01$ ) compared to the vehicle control.

Students t test for unpaired samples.

#### 6.2.4. Effect on alkaline and acid phosphatase enzyme activity.

Alkaline and acid phosphatase enzyme levels in untreated tissue was highly varied ( $p < 0.01$ ) between groups of rats. Within groups of rats the enzyme activities in untreated tissue did not vary. In PHB and P(HB-HV) treated tissue the enzyme activities appeared to significantly differ between groups. This was thought to be due to the actual injection technique of different animal technicians. Thus absolute enzyme levels were difficult to compare between experiments and are best compared to the vehicle controls and untreated tissues within each experiment and the mean value was used as the basis of comparison for the test.

#### Effect on tissue alkaline phosphatase activity.

The activities of tissue alkaline phosphatase after intramuscular injection of the polymers, monomers, the injection vehicle and the sham controls are given in table 6.3 and then presented in table 6.4 as the mean percentage change in enzyme activity when compared to the injection vehicle enzyme activity at any one time after injection.

The test was sensitive even to the sham injection i.e. when no material was injected. However the enzyme activities due to penetration of muscle with the needle were not significantly higher than the levels in untreated muscle on any day due to the large standard deviation within both groups. Injection of vehicle caused a larger but not significant response with respect to sham injected tissue throughout the 10 days and at day 40 there was no difference in enzyme activities between vehicle injected tissue, sham injected tissue or untreated tissue.

Following the injection of the positive control, zymosan (100 $\mu$ l/ml), into the muscle tissue, highly significant increases ( $p < 0.001$ ) in the alkaline phosphatase activity throughout the 10 day



Table 6.3 Tissue alkaline phosphatase activities following intramuscular injection with test materials.

Test	Alkaline phosphatase activity in IU mean (S.D.)					
	Time in days					
	1	2	4	6	8	10
PHB 10mg/ml	67 (10)	309 (60)	72 (26)	128 (28)	115 (32)	87 (0.5)
Vehicle	41 (14)	224 (88)	59 (14)	111 (12)	129 (38)	58 (10)
			Untreated rat control 49 (12)			
P(HB-HV)10mg/ml	337 (22)	448 (92)	854 (204)	510 (86)	591 (160)	* 567 (30)
Vehicle	296 (48)	381 (44)	807 (66)	385 (94)	389 (38)	296 (56)
			Untreated rat control 338 (58)			
HB 10	281 (42)	279 (38)	240 (58)	266 (28)	228 (50)	247 (40)
Vehicle	253 (46)	201 (40)	173 (22)	190 (42)	203 (50)	180 (34)
			Untreated rat control 149 (36)			
HB 100	224 (96)	145 (32)	209 (38)	147 (68)	86 (16)	127 (48)
Vehicle	128 (32)	143 (34)	140 (36)	95 (20)	90 (14)	92 (34)
			Untreated rat control 149 (36)			
HV 10	326 (54)	321 (122)	273 (40)	317 (28)	391 (74)	379 (78)
Vehicle	340 (32)	360 (104)	299 (36)	284 (40)	358 (122)	331 (104)
			Untreated rat control 211 (46)			

HV 100	1103 (248)	1342 (180)	1056 (212)	854 (126)	807 (126)	807 (202)
Vehicle	576 (60)	954 (114)	845 (238)	655 (74)	643 (46)	671 (270)
			Untreated rat control	211 (46)		
Zymosan	**1085 (226)	**973 (46)	**1113 (262)	**780 (148)	**574 (62)	**641 (44)
Vehicle	117 (32)	78 (32)	73 (22)	61 (26)	68 (6)	56 (6)
			Untreated rat control	137 (24)		
P.A.A.	144 (18)	221 (82)	206 (18)	214 (56)	*322 (36)	*356 (24)
Vehicle	149 (16)	206 (14)	211 (20)	177 (16)	216 (26)	202 (60)
			Untreated rat control	163 (8)		
Vehicle	181 (48)	330 (108)	204 (24)	227 (86)	255 (90)	172 (12)
Sham	105 (40)	162 (116)	239 (86)	175 (90)	173 (36)	137 (16)
			Untreated rat control	115 (44)		

\* Statistically significant at the 99% confidence level ( $p < 0.01$ ) when compared to vehicle treated tissue.

\*\* Statistically significant at the 99.9% confidence level ( $p < 0.001$ ) when compared to vehicle treated tissue.

Student's t test for paired samples,  $n = 4$

$n = 14$  for untreated tissue (mean value (S.D.) given over whole time period)

Table 6.4 Alkaline phosphatase activity for each biomaterial expressed as mean % change from vehicle.

	Time in days					
	1	2	4	6	8	10
PHB 10mg/ml	65.12	37.70	22.43	16.00	-11.20	48.96
P(HB-HV) 10mg/ml	12.10	14.96	5.80	32.40	51.97	91.40
HB 10mg/ml	11.09	38.59	39.25	40.21	3.40	37.70
HB 100mg/ml	74.95	1.40	49.35	54.99	-3.80	37.70
HV 10mg/ml	-4.25	-10.60	-8.80	11.67	9.30	14.50
HV 100mg/ml	91.47	40.67	24.90	30.42	25.47	20.27
Zymosan 100ug/ml	826.68	1145.50	1420.70	1171.10	744.81	1034.50
PAA 10mg/ml	-3.53	7.20	-2.30	20.45	49.40	76.10
Sham	-42.30	50.78	17.40	-22.90	-30.40	-20.62

Table 6.5 Tissue acid phosphatase activities following intramuscular injection of test materials.

Test	Acid phosphatase activity in IU      mean (S.D.)					
	Time in days					
	1	2	4	6	8	10
PHB 10mg/ml	499 (102)	433 (62)	695 (170)	612 (114)	428 (24)	497 (44)
Vehicle	334 (72)	330 (48)	556 (118)	608 (80)	389 (26)	542 (56)
			Untreated rat control	337 (36)		
P(HB-HV)10mg/ml	165 (32)	139 (20)	275 (108)	200 (20)	179 (62)	170 (22)
Vehicle	150 (18)	146 (18)	236 (48)	236 (100)	145 (12)	156 (20)
			Untreated rat control	169 (90)		
HB 10mg/ml	297 (68)	262 (30)	315 (24)	227 (16)	270 (4)	246 (2)
Vehicle	183 (78)	205 (46)	236 (8)	203 (100)	242 (56)	219 (10)
			Untreated rat control	171 (28)		
HB 100mg/ml	289 (54)	188 (50)	387 (74)	106 (24)	221 (26)	246 (16)
Vehicle	193 (66)	157 (54)	141 (32)	90 (50)	164 (32)	156 (16)
			Untreated rat control	171 (28)		
HV 10mg/ml	98 (22)	93 (28)	57 (6)	61 (32)	120 (28)	71 (16)
Vehicle	115 (14)	83 (30)	53 (10)	59 (40)	89 (38)	80 (24)
			Untreated rat control	114 (16)		

HV 100mg/ml	296 (56)	198 (14)	233 (50)	238 (62)	314 (70)	351 (56)
Vehicle	180 (20)	135 (32)	257 (34)	182 (54)	272 (82)	292 (52)
			Untreated rat control 114 (16)			
Zymosan	493 (76)	528 (22)	*502 (82)	*626 (110)	*443 (96)	*468 (68)
Vehicle	378 (35)	335 (22)	272 (39)	316 (37)	349 (51)	266 (17)
			Untreated rat control 221 (30)			
PAA	61 (6)	*110 (20)	*113 (54)	*100 (30)	*154 (21)	*205 (102)
Vehicle	52 (6)	45 (14)	48 (18)	42 (10)	60 (14)	52 (8)
			Untreated rat control 55 (4)			
Vehicle	122 (32)	180 (44)	74 (14)	191 (42)	68 (56)	33 (6)
Sham	112 (22)	116 (30)	78 (22)	114 (16)	56 (50)	27 (12)
			Untreated rat control 85 (22)			

\* Statistically significant at the 99% confidence level ( $p < 0.01$ ) when compared to the vehicle control.  
Student's t test for paired samples,  $n = 4$ .  
 $n = 14$  for untreated tissue (mean value (S.D.) given for whole time period.

Table 6.6 Acid phosphatase activity of each biomaterial expressed as mean % change from vehicle.

	Time in days					
	1	2	4	6	8	10
PHB 10mg/ml	49.10	31.50	24.88	0.60	10.00	-8.00
P(HB-HV) 10mg/ml	10.50	-4.90	16.50	-15.60	23.50	9.10
HB 10mg/ml	62.50	28.10	33.40	17.90	11.60	12.30
HB 100mg/ml	49.70	20.00	174.97	17.90	34.70	57.80
HV 10mg/ml	-14.80	1.36	6.90	3.40	34.67	-11.97
HV 100mg/ml	64.16	46.40	-9.25	30.29	15.30	20.22
Zymosan 100mg/ml	30.40	57.30	84.66	97.70	27.09	75.90
PAA 100mg/ml	16.41	142.30	133.60	141.30	157.70	294.70
Sham	9.40	55.00	-5.30	67.40	21.10	21.10

period of examination when compared to the enzyme activities of the vehicle and untreated muscle were evident. PAA (100mg/ml) showed a significant increase in alkaline phosphatase activity at day 8 ( $p < 0.05$ ) and 10 ( $p < 0.001$ ) when compared to the vehicle injected muscle. By day 40 the activity had decreased to control (untreated muscle) levels for both the zymosan and PAA treated muscles.

The PHB treated muscles showed an elevation in enzyme activity two days after injection, this was significantly different ( $p < 0.01$ ) to the enzyme activity in the muscle of untreated rats. A similar significant increase was seen for the vehicle treated muscles although there was no significant difference between polymer and vehicle treatments at this point. From day four to forty there were no significant increases ( $p > 0.05$ ) in the alkaline phosphatase activity in polymer treated muscles when compared to untreated muscle, although the activities tended to be significantly ( $p < 0.01$ ) higher in both cases when compared to untreated muscle. When 10 mg/ml and 100mg/ml solutions of HB were injected no significant increases in alkaline phosphatase activity when compared to vehicle treated rat muscle were seen over a 40 day period. P(HB-HV) injections produced transient acute increases in the alkaline phosphatase activity which were not significantly different from the activity seen in the untreated muscle, but levels remained higher for longer and at day 10 a highly significant increase ( $p < 0.001$ ), in alkaline phosphatase activity was evident. By day 40 this effect had resolved to levels of enzyme activity comparable to the untreated muscle. In common with HB no significant increase in alkaline phosphatase activity were seen when a 10mg/ml solution of HV was injected, when compared to the vehicle treated muscle. When the concentration of HV was raised to 100mg/ml, highly significant increases ( $p < 0.001$ ) in alkaline

phosphatase activity when compared to both vehicle treated and untreated rat muscle were recorded during the first two days following its injection into the muscle.

#### Effect on tissue acid phosphatase activity.

The activities of tissue acid phosphatase after intramuscular injection of identical materials and controls are given in table 6.5 and presented in table 6.6 as the mean percentage change in enzyme activity when compared to the injection vehicle enzyme activity at any one time after injection.

Tissue acid phosphatase activity significantly increased ( $p < 0.01$ ) on days 4-6 after injection of suspensions of PHB microspheres when compared to untreated muscles enzyme activities. Levels of enzyme activity remained higher but not significant when compared to untreated tissues over a 10 day period due to the considerable variation between animals. The mean values of enzyme activity of PHB treated tissue were not significantly different from vehicle injected tissues when examined using a t-test. At both concentrations of the monomer, HB, a highly significant increase ( $p < 0.001$ ) in the tissue acid phosphatase activity on day 4 when compared to vehicle injected tissue and untreated tissue was evident, these elevated levels of enzyme activity were then seen to reduce to the baseline level of activity of untreated tissue during days 6-10.

The copolymer P(HB-HV), did not significantly increase acid phosphatase activity at any time period studied during the experiment when compared to both untreated tissue enzyme activities and injected vehicle tissue enzyme activities. Following injection of the monomer HV (100mg/ml) a significant increase ( $p < 0.01$ ) in acid phosphatase activity when compared to vehicle treated and untreated tissue at day 1 was seen. The 10mg/ml HV solution did not evoke any



significant increases in the acid phosphatase activity during the time course of the experiment. The two positive controls, zymosan and PAA, both produced a significant increase ( $p < 0.01$ ) in acid phosphatase activity from days 4 - 10 when compared to untreated tissue and vehicle injected tissue which was resolved by day 40 in both cases.

#### Serum alkaline and acid phosphatase enzyme activities.

When serum was assayed for increases in the alkaline phosphatase and acid phosphatase activities compared to untreated rats no significant increases were found for any of the treatments, as shown in tables 6.7 and 6.8.

#### Effect on enzyme activity following implantation of films.

Tables 6.9 and 6.10 give the activities of alkaline and acid phosphatase found in the muscle tissue following implantation with polymer films. The results indicate a significant increase ( $p < 0.01$ ) in alkaline phosphatase activity for the control film PMMA at days 6-10 when compared to the sham operated sites, which decreased to sham control levels by day 40. PHB, evoked a significant increase ( $p < 0.01$ ) in alkaline phosphatase activity on days 4 and 10 which had resolved by day 40. The copolymer showed similar significant increases on days 4 and 8 only. Significant increases ( $p < 0.01$ ) in tissue acid phosphatase activity was seen on days 2 and 4 for PHB, days 1, 4 and 6 for P(HB-HV) and days 4, 8 and 10 for PMMA films. Tables 6.11 and 6.12 show no differences in serum enzyme levels for any of the implanted films indicating no serious toxicity or inflammation is due to the polymers.

Data obtained for serum levels of enzymes were unambiguous but the tissue enzyme data were conclusive only for inflammatory

Table 6.8 Serum acid phosphatase activities following intramuscular injection with test materials.

Test	Acid phosphatase activity in IU      mean (S.D.)					
	Time in days					
	1	2	4	6	8	10
PHB/Vehicle	52 (24)	61 (12)	71 (10)	64 (16)	44 (2)	33 (12)
				Control rat 45 (4)		
P(HB-HV)/ Vehicle	37 (18)	33 (6)	65 (16)	37 (14)	34 (8)	36 (10)
				Control rat 26 (16)		
HB 10mg/ml / Vehicle	64 (16)	62 (12)	55 (32)	51 (28)	21 (10)	50 (20)
				Control rat 60 (22)		
HB 100mg/ml/ Vehicle	41 (6)	61 (8)	32 (6)	64 (10)	14 (4)	46 (10)
				Control rat 60 (22)		
HV 10mg/ml/ Vehicle	15 (2)	15 (4)	14 (2)	16 (6)	14 (4)	12 (8)
				Control rat 20 (4)		

HV 100mg/ml/ Vehicle	37 (2)	156 (22)	26 (10)	25 (2)	54 (14)	29 (6)
				Control rat 20 (2)		
Zymosan/ Vehicle	50 (2)	63 (12)	73 (20)	76 (14)	70 (6)	66 (14)
				Control rat 53 (22)		
PAA / Vehicle	14 (1)	12 (2)	20 (20)	30 (18)	20 (5)	18 (4)
				Control rat 17 (4)		
Vehicle / Sham	17 (6)	15 (10)	11 (2)	11 (12)	10 (20)	13 (12)
				(Control rat 12 +/- 10)		

Figures represent mean of 4 samples.

n= 14 for control value (mean value (S.D.) is given for whole time period)

Table 6.7 Serum alkaline phosphatase activities following injection with test materials

Test	Alkaline phosphatase in IU      mean (95% C.I.)    n=4					
	Time in days					
	1	2	4	6	8	10
PHB/ Vehicle	133 (12)	118 (10)	150 (5)	116 (14)	178 (21)	114 (4)
				(Control rat 143 +/- 6)		
P(HB-HV)/ Vehicle	123 (17)	110 (12)	346 (22)	108 (10)	94 (12)	110 (9)
				(Control rat 66 +/- 1)		
HB 10mg/ml Vehicle	222 (17)	205 (9)	284 (15)	274 (6)	254 (4)	220 (12)
				(Control rat 265 +/- 25)		
HB 100mg/ml	250 (31)	188 (20)	153 (9)	256 (16)	292 (11)	262 (13)
				(Control rat 265 +/- 25)		
HV 10mg/ml / Vehicle	53 (2)	40 (6)	47 (4)	50 (8)	52 (1)	37 (1)
				(Control rat 25 +/- 4)		

HV 100mg/ml/ Vehicle	157 (6)	146 (14)	73 (11)	61 (6)	159 (4)	80 (17)
				(Control rat 25 +/- 4)		
Zymosan/ Vehicle	198 (16)	202 (6)	279 (18)	232 (15)	217 (10)	201 (4)
				(Control rat 113 +/- 10)		
PAA/ Vehicle	49 (2)	41 (2)	46 (2)	51 (9)	67 (2)	58 (6)
				(Control rat 49 +/- 6)		
Vehicle/ Sham	52 (11)	49 (12)	44 (5)	53 (21)	48 (8)	40 (19)
				(Control rat 38 +/- 9)		

n=4 for each time point per experiment  
n=14 for control experiments

Table 6.9 Tissue alkaline phosphatase activities following film implantation.

Test	Alkaline phosphatase in IU mean (S.D.)					
	Time in days					
	1	2	4	6	8	10
PHB	392 (110)	330 (62)	*356 (64)	371 (87)	236 (38)	*301 (44)
Sham	537 (137)	272 (30)	215 (22)	366 (56)	212 (52)	199 (29)
P(HB-HV)	299 (23)	557 (65)	* 500 (79)	523 (77)	*567 (29)	579 (132)
Sham	278 (56)	459 (48)	341 (27)	408 (73)	416 (15)	412 (56)
PMMA	247 (24)	313 (34)	335 (84)	598 (33)	907 (60)	718 (88)
Sham	324 (62)	253 (22)	353 (10)	*315 (34)	*624 (99)	*484 (40)

Figures represent the mean (S.D.) of 4 experiments.

\* Significant at the 99% confidence level ( $p < 0.01$ ) when compared to sham sites.

Student's t test for paired samples.

Table 6.10 Tissue acid phosphatase activities following implantation of films.

Acid phosphatase in IU (mean +/- S.D)						
Test	Time in days					
	1	2	4	6	8	10
PHB	273 (70)	223 (13)	*387 (29)	233 (18)	173 (14)	111 (4)
Sham	225 (81)	160 (18)	198 (53)	247 (64)	143 (27)	109 14)
P(HB-HV)	121 (19)	168 (26)	*167 (8)	*196 (27)	163 (26)	163 (10)
Sham	171 (12)	196 (20)	102 (16)	112 (15)	132 (33)	113 (36)
PMMA	213 (34)	211 (12)	*263 (9)	173 (12)	*237 (23)	*215 (21)
Sham	171 (23)	255 (30)	207 (14)	144 (21)	140 (10)	109 (33)

Figures represent the mean (S.D.) of 4 experiments.

\* Statistically significant at the 99% confidence level ( $p < 0.01$ ) when compared to sham sites.  
Student's t test for paired samples.

Table 6.11 Serum alkaline phosphatase activities following implantation with films.

	Alkaline phosphatase in IU      mean (S.D.)					
	<div> <div>←</div> <div>Time in days</div> <div>→</div> </div>					
Test	1	2	4	6	8	10
PHB/Sham	31 (21)	55 (8)	59 (10)	30 (6)	62 (4)	53 (8)
P(HB-HV)/ Sham	41 (7)	63 (3)	38 (22)	31 (22)	35 (7)	39 (7)
PMMA/ Sham	36 (3)	45 (9)	57 (16)	44 (6)	73 (4)	97 (3)

Figures represent the mean (S.D.) of 4 samples.



Table 6.12 Serum acid phosphatase activities following film implantation.

Acid phosphatase in IU mean (S.D.)						
Test	Time in days					
	1	2	4	6	8	10
PHB/ Sham	24 (2)	25 (4)	21 (4)	19 (2)	28 (11)	28 (2)
P(HB-IV)/ Sham	24 (3)	26 (5)	16 (2)	20 (6)	27 (4)	17 (2)
PMMA/ Sham	30 (4)	14 (2)	23 (5)	16 (4)	27 (2)	31 (4)

Figures represent mean (S.D.) of 4 samples.

materials such as zymosan. The interpretation of data relevant to the implantation of PHB and P(HB-HV) was difficult due to inherent variability of the animal study, rather than the sensitivity of the assay. For this reason a second study was conducted with the aim of testing the effects of PHB and P(HB-HV) microsphere injections using a series of different batches of each polymer. Great care was taken during this study to ensure that the injections were performed in as reproducible a manner as possible. The results of this study are given in tables 6.13 -6.14.

The injection of two different batches of PHB microspheres into rat muscle tissue produced similar patterns in enzyme activities (table 6.13), to those seen in the initial experiments. Significant increases ( $p < 0.01$ ) in alkaline phosphatase activity were seen on days 1-2 when compared to untreated muscle tissue, but no significant increase was seen when compared to the vehicle treated muscle. Acid phosphatase activity was significantly increased ( $P < 0.01$ ) on day 6 in PHB injected muscle when compared to the vehicle injected muscle. As indicated by a large S.D. from the mean in this second set of experiments there still appears to be an inherent variability of the study.

The three separate batches of P(HB-HV) also produced similar increases in enzyme activities over the test period. However the results of this experiment differed slightly to those obtained in the first set of experiments. Significant ( $p < 0.01$ ) increases in alkaline phosphatase when compared to vehicle treated muscle were seen on days 6-14 whereas a significant increase in activity was seen on day 10 only in the first set of experiments. Acid phosphatase was also significantly increased ( $p < 0.01$ ) on days 8-14 when compared to untreated muscle, whereas no significant increases were detected in

Table 6.14. Tissue acid/alkaline phosphatase activities following i.m. injection of various batches of P(HB-HV).\_

	Alkaline Phosphatase activity						mean (S.D) n=4		
	Time in days								
	1	2	4	6	8	10	14	21	40
Batch 1	469 (74)	410 (52)	474 (102)	*646 (12)	*599 (38)	*787 (21)	*853 (21)	425 (31)	263 (14)
Vehicle	409 (53)	324 (20)	423 (37)	311 (42)	312 (22)	351 (40)	395 (55)	325 (67)	243 (18)
Batch 2	547 (54)	409 (175)	529 (115)	*1066 (71)	*762 (33)	*813 (58)	*879 (30)	483 (60)	359 (24)
Vehicle	590 (38)	444 (52)	449 (71)	465 (57)	375 (40)	345 (19)	363 (11)	307 (58)	321 (15)
Batch 3	509 (171)	508 (175)	464 (100)	*878 (61)	*859 (43)	*883 (20)	*1024 (65)	458 (28)	312 (23)
Vehicle	325 (66)	316 (33)	379 (60)	359 (16)	326 (20)	318 (38)	343 (21)	372 (44)	255 (27)

	Acid phosphatase activity						mean (S.D)	n=4	
	1	2	4	6	8	10	14	21	40
Batch 1	334 (6)	362 (41)	325 (27)	442 (47)	*642 (53)	*760 (21)	*660 (36)	370 (18)	298 (24)
Vehicle	332 (21)	288 (20)	308 (20)	362 (31)	266 (19)	216 (37)	285 (10)	321 (16)	206 (45)
Batch 2	300 (26)	361 (15)	376 (72)	497 (55)	*762 (46)	*759 (30)	*702 (68)	319 (31)	287 (31)
Vehicle	271 (27)	294 (18)	301 (37)	392 (41)	229 (35)	233 (21)	207 (19)	242 (14)	215 (17)
Batch 3	337 (26)	370 (37)	339 (44)	436 (20)	*695 (32)	*732 (33)	*694 (16)	287 (29)	306 (29)
Vehicle	287 (13)	264 (58)	272 (25)	320 (19)	343 (15)	360 (15)	224 (29)	194 (40)	263 (17)

\* Statistically significant at the 99% confidence interval ( $p < 0.01$ ) using a student's t test for paired samples.

Table 6.13 Tissue alkaline/acid phosphatase activities following i.m. injection of two batches of PHB.

		Alkaline phosphatase activity						mean (S.D)	n=4	
		1	2	4	6	8	10	14	21	40
Batch 1		*298 (17)	*349 (5)	323 (25)	357 (17)	521 (12)	425 (81)	491 (63)	200 (66)	212 (45)
Vehicle		187 (7)	256 (19)	264 (46)	299 (51)	423 (66)	399 (76)	379 (25)	167 (11)	184 (12)
Batch 2		*320 (16)	*353 (35)	412 (48)	406 (39)	472 (30)	409 (63)	465 (34)	316 (20)	254 (19)
Vehicle		223 (11)	249 (11)	311 (52)	368 (57)	421 (10)	391 (17)	395 (37)	278 (21)	265 (13)

		Acid phosphatase activity						mean (S.D)	n=4	
		1	2	4	6	8	10	14	21	40
Batch 1		336 (7)	319 (26)	366 (34)	*612 (38)	484 (20)	414 (13)	460 (23)	344 (16)	390 (38)
Vehicle		315 (35)	298 (15)	342 (29)	416 (41)	423 (18)	440 (21)	395 (12)	338 (10)	303 (23)
Batch 2		347 (12)	375 (45)	398 (16)	*573 (30)	557 (85)	439 (69)	454 (61)	591 (72)	421 (41)
Vehicle		351 (37)	312 (26)	368 (39)	363 (21)	455 (59)	346 (24)	499 (80)	490 (47)	401 (53)

\* Statistically significant at the 99% confidence interval ( $p < 0.01$ ) using a student's t test for paired samples.

the first set of experiments. The results of this second set of experiments appear to more closely correlate with the findings of the histology of P(HB-HV), where a florid reaction was observed 7 days after injection, however large S.D. also indicate an inherent variability in the study.

### 6.3. Discussion.

Stimulation of the inflammatory response is often the first response to tissue damage or to the presence of foreign material, which can then develop to involve the immune system. Implantation of any material, (inert, naturally occurring, or foreign) is likely to stimulate a response due initially to its physical presence. Whether the response develops into a significant inflammatory reaction or results in toxicity will depend on the material in question - the biocompatibility of the implant being a function of the tissue response. The polymers under study were not believed to be acutely toxic, with reference to their compatibility with cells in culture (chapter 3) and previous work (Korsatko *et al.*, 1983), but should significant or prolonged inflammation result from their use, they would be unacceptable for use in clinical practice. This study set out to assess their biocompatibility *in vivo*.

The magnitude and duration of the inflammatory response has been used, for a number of years by many workers in the establishment of a materials biocompatibility, (Turner *et al.*, 1973; Marchant *et al.*, 1983,1984). The most common methods of assessment have been qualitative histological studies. Histology is an essential component in the evaluation of tissue compatibility and the implanted material. Such techniques have been particularly useful initial evaluation of the material. A number of implantation sites have been used to assess material biocompatibility. Langer *et al.*, (1981) studied the biocompatibility of a number of polymeric delivery systems for macromolecular drugs using the cornea as the implantation site. However this site has the disadvantage of avascularity and therefore the haemodynamic changes that occur with tissue damage cannot be

seen, perhaps leading to false conclusions of the materials biocompatibility. Other sites for implantation used in the study of *in vivo* biocompatibility include the rat middle ear as used by Williams and Blayney (1986) and Bakker *et al.*, (1988) and the bone, as used by Mattie and Bajpai (1988) for assessment of materials for use in bone grafting. Subcutaneous implantation sites have also been used (Leong *et al.*, 1986; Marion *et al.*, 1980) but again this tissue is not ideal for histology or enzyme histochemical studies as it is poorly vascularized and is extremely fatty. Perhaps the best mode of implantation for assessment of biocompatibility is intramuscularly into the gluteal or gastrocnemius muscle of the rat or rabbit. This has been used by many workers including Marchant *et al.* (1983, 1984, 1985, 1988) who used a cage implant system. This intramuscular site of implantation is suggested in the British Standards for testing of Biocompatibility (BS 5736 Part 2, 1981) by the ATMS (F763-82, 1982) and the U.S. Pharmacopeia (section 661, 1980).

Histological assay of tissue adjacent to an implant demands particular attention to implantation and biopsy techniques and although such evaluations are necessary they remain subjective to a degree and difficult to quantitate. The histological evaluation of PHB, P(HB-HV) and their degradation products revealed that only the copolymer gave rise to continued inflammation over 14 days. Why P(HB-HV) resulted in a florid reaction when PHB did not cannot as yet be explained. Korsatko *et al.*, (1984) assessed the degree of inflammation caused by tablets of PHB implanted subcutaneously into the neck crease of mice. Assessment was carried out by histological examination. A mild foreign-body reaction in the form of inflammatory processes in the region of the implant lasted throughout the 20 week implantation period. A cell rich connective tissue capsule which was

highly vascularised developed after five weeks implantation. These results are not unlike our own, as only a transient inflammatory reaction was seen with the injected PHB microspheres, suggesting that the physical form of the PHB tablet had caused prolonged inflammation. However in this study PHB films were not seen to prolong the initial inflammatory reaction associated with the surgical trauma of implantation. It is believed that subcutaneous implantation into the neck crease of mice used by Korsatko and his coworkers to assess the tissue reaction to PHB would not give enough information to the actual inflammatory potential of the material as this is a fatty and poorly vascularised tissue. PAA has been used in other histology assessments (Langer *et al.*, 1981) where it was found as in this work to produce significant inflammation. This may be explained by the leaching of monomers, methacrylamide, or initiators of polymerisation which are potentially toxic. The PHB and P(HB-HV) films were well tolerated by the soft tissue and all indications were of good biocompatibility exemplified by an intact local blood supply, thin fibrous capsule and good cellularity, the features as defined by Coleman *et al.*, (1974) for good biocompatibility.

Damage to a vascularized tissue leads to increased permeability, an outpouring of exudate fluid, blood cells and plasma cells from the vessels and into the extravascular territory. Vascular permeability and exudate changes are mediated by the local release of active endogenous chemical substances including histamine, prostaglandins, kinins, complement derivatives and some lysosomal derivatives, (Bult and Herman, 1985). Vascular permeability changes were assessed by Evans Blue leakage. The responses to the polymers used in this study were negligible (Table 6.2.) suggesting that these materials may be suitable for *in vivo* use. Raised permeability was



observed after injection of HB and HV and was thought to be related to an osmotic effect as the results were not significantly different to sucrose on a molar basis. Zymosan used as a positive marker for measurement of vascular permeability changes (Williams, 1979; Wedmore and Williams, 1981) caused substantial vascular permeability which was not due to pH effects caused by the irritants HCl and NaOH (pH 0.45 and 12.84 respectively). However histamine and 5HT, both commonly used to induce increases in the permeability of the surrounding vasculature (Arvier *et al*, 1977; Coughlin *et al*, 1981), did not significantly increase vascular permeability when compared to the vehicle of polymer injection suggesting the inflammatory potential of the vehicle itself.

The quantitative study of cellular dynamics in relation to implant toxicity has certain definite advantages over procedures relying solely on observed macroscopic or microscopic observations for histocompatibility. The results are not subjective and are based on cellular function and behaviour and not merely on cell population or morphology. The PMN alkaline phosphatase and macrophage acid phosphatase lysosomal activity adjacent to the tissue appears to be related to the toxicity of the implant components. These enzymes can be measured quantitatively therefore giving an objective parameter for application to the study of cellular toxicity of materials implanted *in vivo*. Salthouse and his coworkers (1969, 1970, 1975) have extensively reviewed enzyme histochemistry in the study of biocompatibility and these techniques have since been used by many workers (Goldman and Raz, 1975; Allison *et al.*, 1969; Morland and Morland, 1978; McNamara and Williams, 1984).

The results of this study suggest that intramuscular injection of PHB or P(HB-HV) provoke a mild acute inflammatory response which

is characterized by the influx of PMN's into the vicinity of the implant and hence an increase in alkaline phosphatase activity. Alkaline phosphatase is located within the specific granule of the PMN's and its presence in the exudate denotes its release either by the death of the PMN with lytic release or through an exocytosis mechanism that involves non-cytolytic extracellular release of this enzyme from the granules, usually occurring during phagocytosis. This acute inflammatory response did not differ significantly from the vehicle injected rats and was only transient. There were some differences in the duration of the acute inflammatory effect, particularly in P(HB-HV) injected rat muscle as indicated by significant increases in alkaline phosphatase activity on days 6-14 seen in the second set of experiments. It is not clear at present how important these effects may be. A sustained PMN concentration as shown by the zymosan test which produced continued elevation of the enzyme was an indication of continued PMN migration from the vasculature to the injection/implantation site and suggested that inflammatory mediators were being maintained at relatively high concentrations in the inflammatory exudate. The subsequent attraction of macrophages into the inflammatory site through the chemotactic factors released by the PMN's and other blood cells in the vicinity, was indicated by a clear increase in acid phosphatase activity after injection of zymosan. As the macrophages attempt to phagocytose foreign material, exocytosis of lysosomal enzymes, of which acid phosphatase is the most abundant, occurs. Significant increases in acid phosphatase activity was also seen on days 8-14 in P(HB-HV) treated muscle, this correlated well with the increased PMN concentration and alkaline phosphatase activity seen at this time, when macrophage attraction through the chemotactic factors released

by PMNs probably occurred.

Salthouse equated high acid phosphatase activity with cell lysis and tissue destruction. The intensity and duration of the inflammatory response measured through these enzymes is controlled by the mediators determined by the injected material, site of injection and the reactive capability of the host. As indicated by the experiments conducted in this study inflammation caused by PHB or P(HB-HV) was transient and was also observed after injection of vehicle and, to some extent, by simple penetration of the needle. Thus it may be that the observed inflammation was due to the trauma of injection and not to the polymers or monomers themselves. The short interaction shown by the macrophages representative of later stages of inflammation, indicated that the tissue's responses to the polymers and monomers were simply those of short duration inflammatory reaction followed by normal wound healing. Highly irritating implants greatly increase acid phosphatase activity, as seen with PVC samples containing organotin stabilizers and phthalate ester plastisizers, (Salthouse *et al.*, 1973; Salthouse and Matlaga; 1975) cat gut suture (Newcombe, 1972), and many metals used in implants. Biocompatible polymers eg. high density medical grade polyethylene and polypropylene evoke only minimal levels of enzyme activity which diminish to undetectable levels after the third or fourth week following implantation, (Spilizewski *et al.*, 1987). The effects of PHB and P(HB-HV) were comparable to those of the latter polymers.

Formulation, geometry and surface characteristics can affect both the tissue reaction and enzyme activity associated with the sample as shown by many workers (Van Oss and Gillaman, 1972; Andrade, 1973; Matlaga *et al.*, 1975; Taylor and Gibbons, 1983; Schakenraad *et*

*al.*, 1987). As suggested by Henson (1980) the inflammatory response is also dependent upon the size of the implant and whether a material is in particulate or bulk form. The former may allow phagocytosis which may provoke a different degree of inflammation than the same material in a non-phagocytosable form such as a film, where frustrated phagocytosis and exocytosis will probably prevail. PHB and P(HB-HV) films, when compared to a biocompatible material, PMMA, did not significantly differ in the enzyme activity produced and any significant inflammation appeared to be due to the surgical procedure of implantation and not the polymers.

In conclusion this study has shown that PHB and P(HB-HV) are biocompatible in the sense that implantation in / adjacent to the rat thigh results in no significant chronic inflammation. On degradation to the monomers, both HB and HV are probably removed and enter the bloodstream prior to excretion. Hence no significant accumulation of breakdown products would be expected. Should the monomers remain at the polymer site this study has suggested that no chronic inflammatory response would result. These polymers are therefore biocompatible within soft muscle tissues.

#### 6.4. Conclusions.

- 1) The tests were sensitive as trauma due to injection was detectable.
- 2) Inflammatory materials such as zymosan produced significant increases in the vascular permeability and clearly raised enzyme levels over a 10 day period.
- 3) There was considerable variability in enzyme activities between animals in both sets of experiments. Often raised levels of enzyme were determined but the S.D. was large leading to the conclusion that there were no significant differences in enzyme activities between polymer treated and vehicle treated rat muscle. Generally an acute effect was evident which had subsided after a few days and certainly after 40 days.
- 4) Rats were found to tolerate large quantities of HB and HV, far more than would be expected at the site of implantation of PHB or P(HB-HV).

CHAPTER 7. Evaluation *in vitro* and *in vivo* of PHB as a biodegradable drug delivery system.

7.1. Introduction.

Interest in controlled-release technology has increased steadily over the last twenty years. Recently major advances in genetic engineering have enabled large quantities of potent polypeptides and proteins to be produced. Practical and effective formulations are now required for their delivery. Polypeptides e.g. insulin and growth hormones are normally administered parenterally, as they are rapidly degraded and deactivated by proteolytic enzymes in the gastro-intestinal tract. However problems are encountered with parenteral administration in that polypeptides have short half-lives and therefore frequent injections are usually required to produce effective plasma concentration (Hutchinson and Furr, 1987; Tice *et al.*, 1989). To overcome this problem drug delivery devices which degrade within the body, have pre-determined life spans and which protect the labile drug during its lifetime are now being developed. Such delivery devices must meet requirements in mechanical properties, biodegradation kinetics, tissue compatibility, drug compatibility and ease of manufacture.

Biodegradable polymers have been used in the development of drug delivery systems for a number of drugs including narcotics, antimalarials and antibiotics, and their applications have been reviewed (Wood, 1980; Heller, 1980; Heller, 1984, Heller *et al.*, 1985; Holland *et al.*, 1986; Davis and Illum; 1986). Early designs based on biodegradable polymers have made use of homo and copolymers of lactic and glycolic acid. The first demonstration of the utility of polylactide, as a biodegradable implant capable of sustained release of a therapeutic agent, was described about 20 years ago

(Yolles *et al.*, 1970). Since then many publications have described the sustained release of pharmaceuticals from P(LA-GA) systems (Woodland *et al.*, 1973; Yolles *et al.*, 1975; Wise *et al.*, 1979; Gilding and Reed, 1979; Wakiyama *et al.*, 1981, 1982; Juni *et al.*, 1985). More recently P(LA-GA) systems have been used for the sustained drug delivery of peptides (Sanders *et al.*, 1984, 1986, 1988; Hutchinson and Furr, 1987). Previous work on these polymers as suture materials has shown that they are inert and biocompatible within the physiological environment after implantation (Kulkarni *et al.*, 1966, 1971; Frazza and Schmitt, 1971; Cutright and Hunsuck, 1971; Gourlay *et al.*, 1978). P(LA-GA) systems degrade to toxicologically acceptable products which are already present in the body, i.e. glycolic acid and lactic acid. Such polymeric carriers are expected to be ideal for formulation of controlled drug delivery systems.

The release of the therapeutic agent from a degradable delivery system will be influenced by the mechanism and rate of degradation of the polymeric carrier. The majority of reports relating to such drug delivery systems concentrate on the degradation of the polymeric carrier by hydrolysis in the absence of enzymes. Heller (1984) classified two extreme types of hydrolysis, bulk hydrolysis and surface hydrolysis. In bulk hydrolysis, chain cleavage takes place throughout the bulk of the material while in surface hydrolysis the reaction is confined to the outer surface of the solid device.

Research has shown that polymers of lactate and glycolate degrade by bulk hydrolysis (Heller, 1986). Monolithic devices which undergo a bulk erosion process often exhibit complex drug release kinetics because release rate is determined by drug diffusion

phenomena and polymer hydrolysis. In general the initial kinetics of release are comparable with those resulting from diffusion from non-erodable monolithic devices; release rate is often proportional to the square root of time as described by Higuchi's equation (Higuchi, 1961). However, as bulk hydrolysis proceeds, the permeability of the polymer increases and thus the rate of diffusion of the drug increases (Heller, 1986; Hadgraft and Guy, 1987).

The kinetics of drug release from monolithic devices which undergo surface hydrolysis are more predictable. Release additional to that expected by matrix diffusion would only occur from the surface of the polymeric carrier as surface erosion of the carrier proceeded. Such devices should be capable of providing zero-order drug release if the drug is entrapped in the polymer matrix and the surface area of the device remains constant. In practice the rate of drug release is likely to decrease with reduction of the total surface area of the device, which will be a consequence of the erosion process. However in principle, by controlling the geometry of the device one could determine its lifetime. For instance, for a laminate the lifetime of the device would be directly proportional to its thickness. Much research is therefore directed towards finding a biocompatible, biodegradable polymer which degrades by surface erosion only. Heller and his colleagues (1985) have developed bioerodible polymers that do undergo surface erosion and are capable of releasing drugs by zero-order kinetics; namely, polyorthoesters. Polymers from this group have been prepared synthetically by the Alza corporation (USA) and are marketed under the trade name Alzamer. Recent work at the university of Bath has shown that the polyesters PHB and P(HB-HV) degrade by surface erosion. (Majid, 1988). The rate of hydrolysis was very slow but could be enhanced by making the



hydrolytic medium more alkaline.

The controlled delivery of a model drug from PHB, P(HB-HV), PLA and P(LA-GA) matrices, produced by melt extrusion, has been investigated. Delivery of proteins and peptides was of particular interest. However proteins degrade to amino acids and are recycled into other body proteins. Neither the original protein nor its metabolites are excreted and it is therefore difficult to measure the absolute release rates of such macromolecules *in vivo* (Brown *et al.*, 1983). Indirect measurement of release rates *in vivo* could be determined by assaying the mass of protein remaining in the implants at various time points, but this would require a large number of animals and expenditure for each investigation. Inulin, a polysaccharide (Mw 5200) was chosen as a model macromolecular drug as it is totally excreted (Smith, 1956; Gutman, 1965) allowing its release kinetics from polymers to be studied *in vivo* (Langer *et al.*, 1981). Release of inulin was determined both *in vitro* and *in vivo* to allow comparison of the respective release kinetics.

Release was studied *in vitro* in both accelerating conditions and physiological conditions. The effect of drug loading on the kinetics of release was studied *in vivo* and *in vitro* and the effect of temperature and pH on kinetics of release was studied *in vitro*.

## 7.2.Materials

Inulin; Sigma Chemicals Co, Poole, Dorset.

$^{14}\text{C}$  Inulin; Sigma Chemicals Co, Poole, Dorset.

Dulbecco's PBS, Oxoid Ltd, England.

Sodium hydroxide pellets; Aldrich Laboratories, Gillingham, Dorset.

Phenol; Aldrich Laboratories, Gillingham, Dorset.

Concentrated sulphuric acid, Fisons Ltd., Loughborough, Leicester.

LKB Optiphase Liquid Scintillation cocktail; FSA Laboratory supplies, Loughborough, Leicester.

LKB Wallac Internal Standard Kit for liquid scintillation counting; FSA Laboratory Supplies, Loughborough, Leicester.

PHB, Mwt 23,000; Marlborough Biopolymers, Teeside.

P(HB-HV), Mw 760,000, 12.6 mole%, Marlborough Biopolymers, Teeside.

Poly (L-lactide) Resomer\* L206; Boeringher Ingleheim, West Germany.

Poly D,L-lactide-co-glycolide 50:50, Resomer\* RG 505; Boeringher Ingleheim , West Germany.

\* Trade name.

## Equipment

LKB Wallac 1215 Rackbeta Liquid Scintillation Counter, Wallac Oy, Finland.

Pye Unicam pu8610 uv/vis kinetics spectrophotometer; Philips, Fisons, Loughborough, Leicester.

### 7.3 In vitro release experiments.

#### 7.3.1. Matrix preparation.

Appropriate amounts of PHB and inulin powders were mixed thoroughly using a pestle and mortar to achieve homogeneity. 100mg of the powder blend was carefully poured into a thermostatically controlled pre-heated die. The mix was melted at 180°C +/- 5°C (PHB blends) for approximately 4 minutes and the melt allowed to cool by ~20°C when the melt could easily be extruded from the die. The extruded rod was cut into sections to give final dimensions 1cm x 0.3mm (length x diameter). P(LA-GA) 50:50 and PLA systems were prepared in essentially the same way but the temperature of melt differed. PLA was successfully extruded at 130°C and P(LA-GA) copolymer at 115°C. P(HB-HV) matrices could not be fabricated with the high molecular weight copolymer using the melt extrusion technique as it was too viscous at melt temperature.

#### 7.3.2. Experimental.

Three 1cm x 0.3mm sections of polymer/inulin extrudate were placed into 20ml closed screw-capped vials with 10ml of sterile release medium; either PBS (pH 7.4) or 0.1M NaOH (pH 12.9). The vials were agitated in a shaking water bath at 37°C or 70°C. 200ul of the buffer solution was removed daily, assayed for inulin content and replaced by 200ul of fresh sterile release media. Sink conditions were ensured due to the large volume of release media relative to the small amount of inulin incorporated into the melts.

At the end of a selected release period, typically 14 days, the extrudate was carefully removed to prevent splintering or disintegration, dried and weighed. Finally the extrudates were examined using scanning electron microscopy.

### 7.3.3. Inulin assay

The assay of inulin used a phenol-sulphuric acid assay commonly used for determination of polysaccharides. This is a non-specific method for detection of neutral carbohydrates and has sufficient sensitivity to measure 1-60ug glucose in 200ul solution (~30uM-2mM).

200ul samples of release media (containing up to 60ug of carbohydrate) were mixed with 200ul of 5% w/v phenol solution in water. 1.0ml of concentrated sulphuric acid was added rapidly and directly to the solution surface without touching the sides of the tube. The solutions were left undisturbed for 10 minutes before vortexing for one minute. A highly coloured product was formed which was assayed spectrophotometrically at 490nm after 30 minutes. The readings were converted to concentration of inulin using calibration curves of inulin in PBS (pH 7.4) or 0.1M NaOH (pH 12.9).

## 7.4. In vivo release experiments.

### 7.4.1. Matrix preparation.

$^{14}\text{C}$  labeled inulin powder was mixed with unlabeled inulin powder and then blended with polymer powder to give drug loadings of 20%w/w in PHB/inulin extrudates and 10%w/w in PLA, P(LA-GA)/inulin extrudates. The polymer/inulin matrix was then extruded through a pre-heated die as described in 7.3.1. PHB/inulin extrudates were steam sterilised at 121°C for 15 minutes before implantation and PLA, P(LA-GA) extrudates sterilised by alcohol washes prior to implantation into the thigh muscle of rats.

### 7.4.2. Implantation technique.

Three polymer/ $^{14}\text{C}$  inulin extrudates, 1cm x 0.3mm were implanted into the thigh muscle of each male Wistar rat (weight 200g) by making a

2cm incision in the gluteal muscle. A pair of sterile round edged scissors was used to create a pocket in the intra-muscular tissue and the sterile extrudates placed into the pocket with sterile forceps. The wound was closed with animal clips. The rats were individually housed in metabolic cages for the duration of the experiment (28 days) after a post-operative recovery period of 3 hours. Urine was collected daily in 15ml graduated collection tubes, allowing urine volumes to be read directly.

#### 7.4.3. Inulin assay for determination of release kinetics *in vivo*.

A 500ul aliquot of the urine collected daily from each rat was added to 500ul of distilled water and 10ml of scintillation fluor in a glass scintillation vial. The radioactivity present was measured using a liquid scintillation counter the cocktail being counted for 120 seconds. A quench correction curve was constructed using a series of  $^{14}\text{C}$  quench standards and this curve was used to correct all counts. Cumulative % release was plotted versus time. After 4 weeks the experiment was terminated and the rats killed. The implants were removed and each site examined visually for host response. Recovered implants were inspected for deterioration weighed and surface erosion/porosity was examined using SEM.

## 7.5. Results.

### 7.5.1. In vitro release kinetics.

Figures 7.1-7.6 show the effects of temperature, pH and drug loadings on the release kinetics of inulin from the drug/polymer matrices. The % cumulative release of inulin is plotted against time. Data plotted is the mean of 15 replicate release studies from each extrudate, standard deviation being < 5% of the mean. The studies were conducted for up to 14 days. Table 7.1 summarises the total percent polymer weight loss and total percent drug loss over 14 days for each variable examined.

Each study exhibited two phases of release. There was an initial burst seen on day 1 in which 5-10% of the incorporated inulin was released. The burst effect was followed by a second phase when approximate zero-order release kinetics were seen for the PHB/inulin extrudates under release conditions of pH 7.4 and temperatures of 37°C and 70°C. Approximate zero-order release kinetics were seen over the first 7 days for the PHB/inulin extrudates under release conditions of pH 12.9 and both temperatures. First order release kinetics were observed for all PLA and P(GA-LA)/inulin extrudates under pH 12.9 and 37°C and 70°C release conditions.

The release of 10% and 15% inulin loaded PHB (Mw 23,000) at 37°C and pH 7.4 is compared in figure 7.1. Over the 14 day release period, both the 10% and 15% inulin loaded PHB extrudates, delivered 12% of the total incorporated inulin. The burst effect occurred on day 1 for both matrix systems delivering 5-6% inulin which was probably due to surface inulin and inulin at the cut edges of the extrudate. The second release phase showed a lower release rate but of approximate zero order kinetics. Figure 7.2. shows the release of inulin from the same extrudates at 70°C, pH 7.4. A similar burst

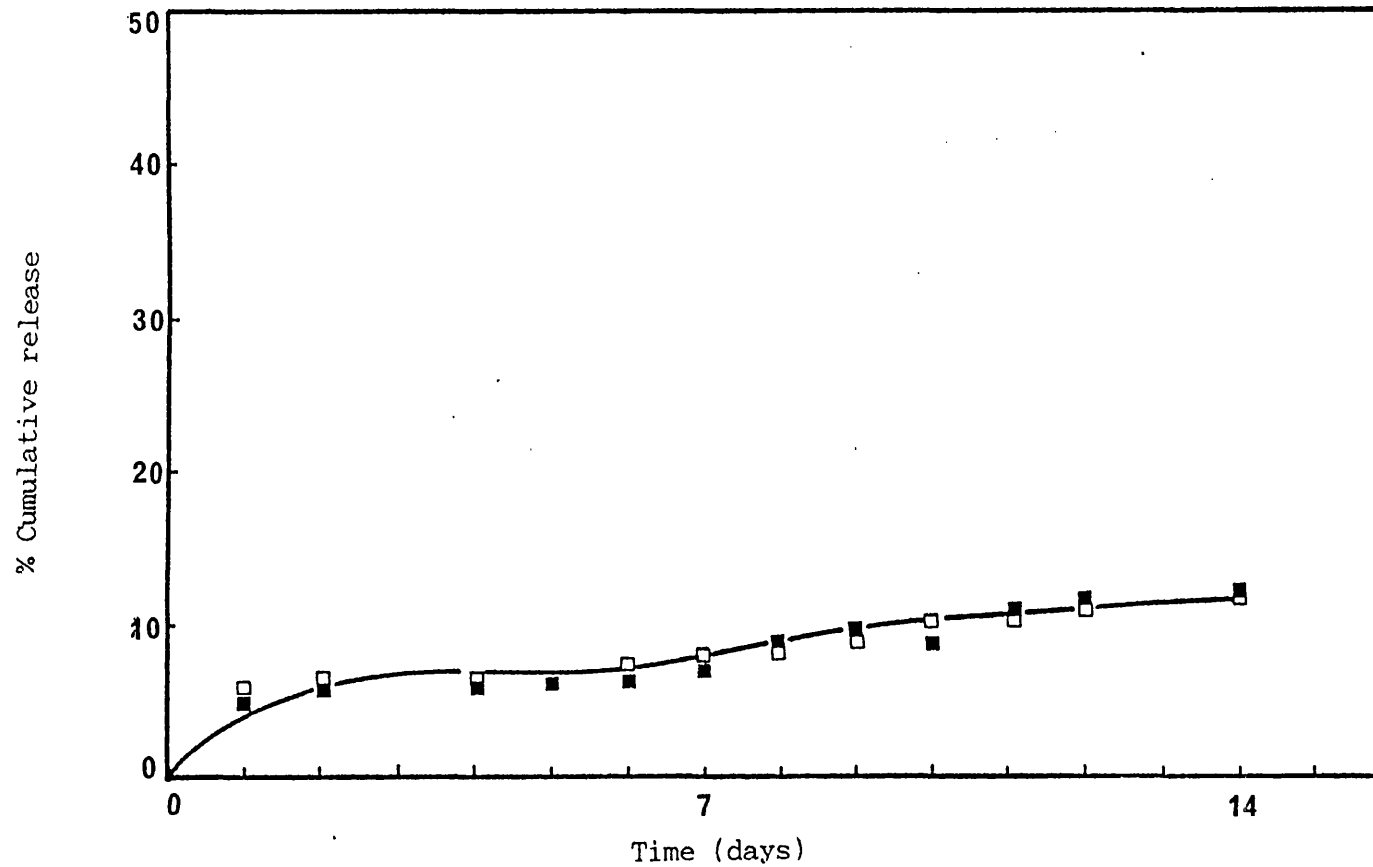


Figure 7.1. Cumulative percent release of 10% and 15% loaded inulin/PHB matrices versus time in days into phosphate buffered saline, pH 7.4 at temperature 37°C.

Points represent the mean release of 15 samples, S.D. <5% of the mean.

■ 10% inulin loading.

□ 15% inulin loading .

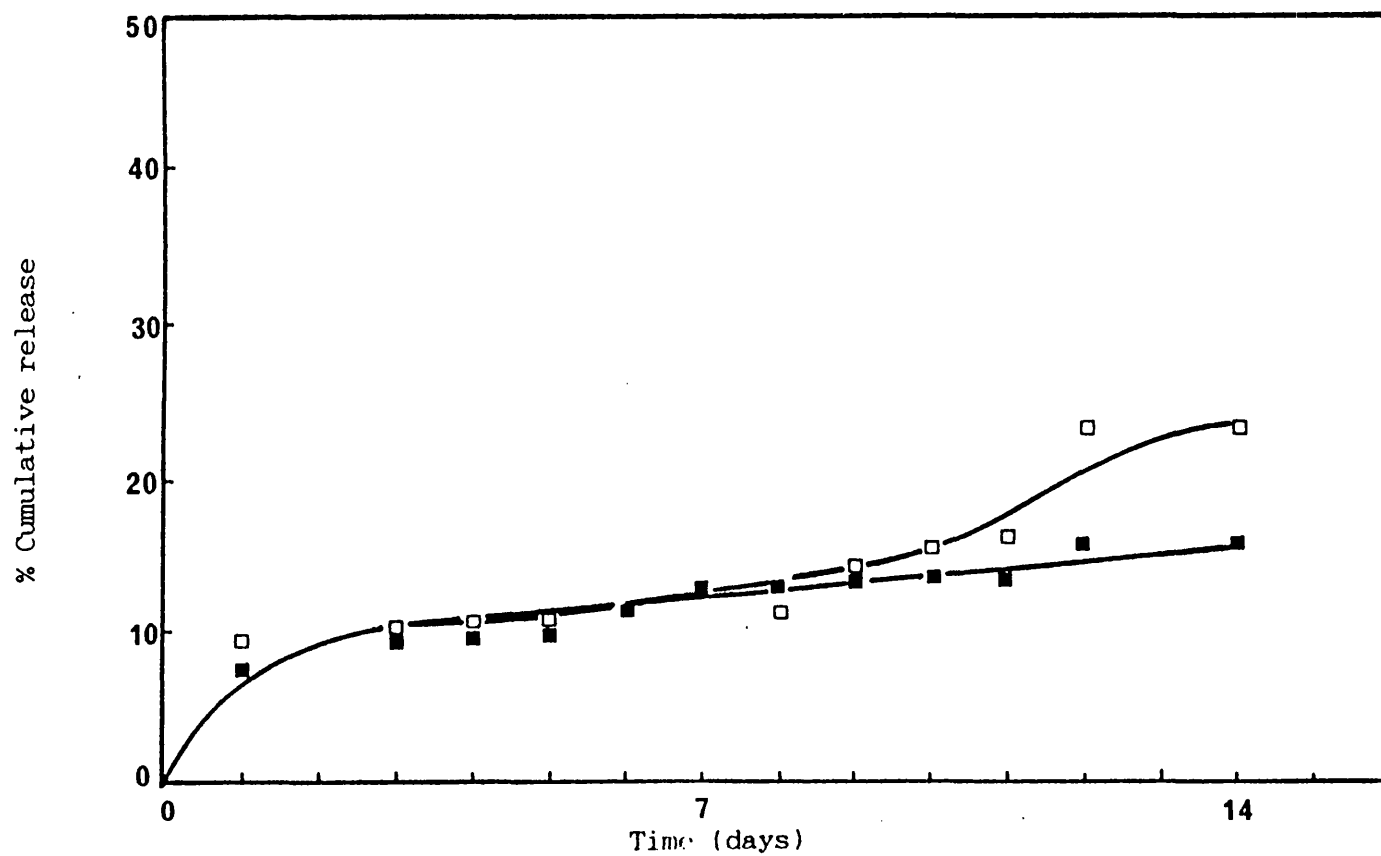


Figure 7.2. Cumulative percent release of 10% and 15% loaded inulin/PHB matrices versus time in days into phosphate buffered saline, pH 7.4 at temperature 70°C.  
 Points represent the mean release of 15 samples, S.D. <5% of the mean.  
 ■ 10% inulin loading.  
 □ 15% inulin loading.



effect was seen on day 1 where 7.5-9.5% of inulin was released. From day 2 to 14 a further 8.5% and 13.5% inulin was released following approximate zero-order release kinetics from the 10% and 15% loaded PHB matrices respectively. As was expected at higher temperatures the amount of inulin released from both the 10% and 15% loaded PHB matrices increased.

The release of inulin from PHB matrices at pH 12.9 and 37°C is shown in figure 7.3. Over the 14 day release period 33.5% and 28% of inulin from the 10% and 15% inulin loaded matrices respectively had been released. An initial burst effect was seen on day 1 when 7% of the total incorporated inulin was released. Over the first week the release of inulin was minimal (<10%). This was followed by a rapid increase in release from day 7 to day 14, following approximate first order kinetics.

Figure 7.4. shows the release at pH 12.9 and 70°C. Over the 14 day release period 32% and 33% of inulin were released from the 10% and 15% inulin loaded matrices. No obvious burst effect was seen and the release of inulin under these conditions appeared to follow first order kinetics.

Table 7.1 shows the polymer weight loss which had occurred through degradation of the polymer matrix and the amount of drug that was released after 14 days. Polymer degradation at pH 12.9 and 70°C was ~ 50% and the drug release was ~32%. This suggests that in order for drug release to occur polymer degradation had to occur. However in all other cases examined the % polymer loss through degradation was less than the % drug release suggesting that release of the drug did not exclusively rely on polymer degradation.

Release rates from PLA and P(LA-GA)/inulin extrudates at pH 12.9 at 37°C and 70°C are shown in figures 7.5 and 7.6

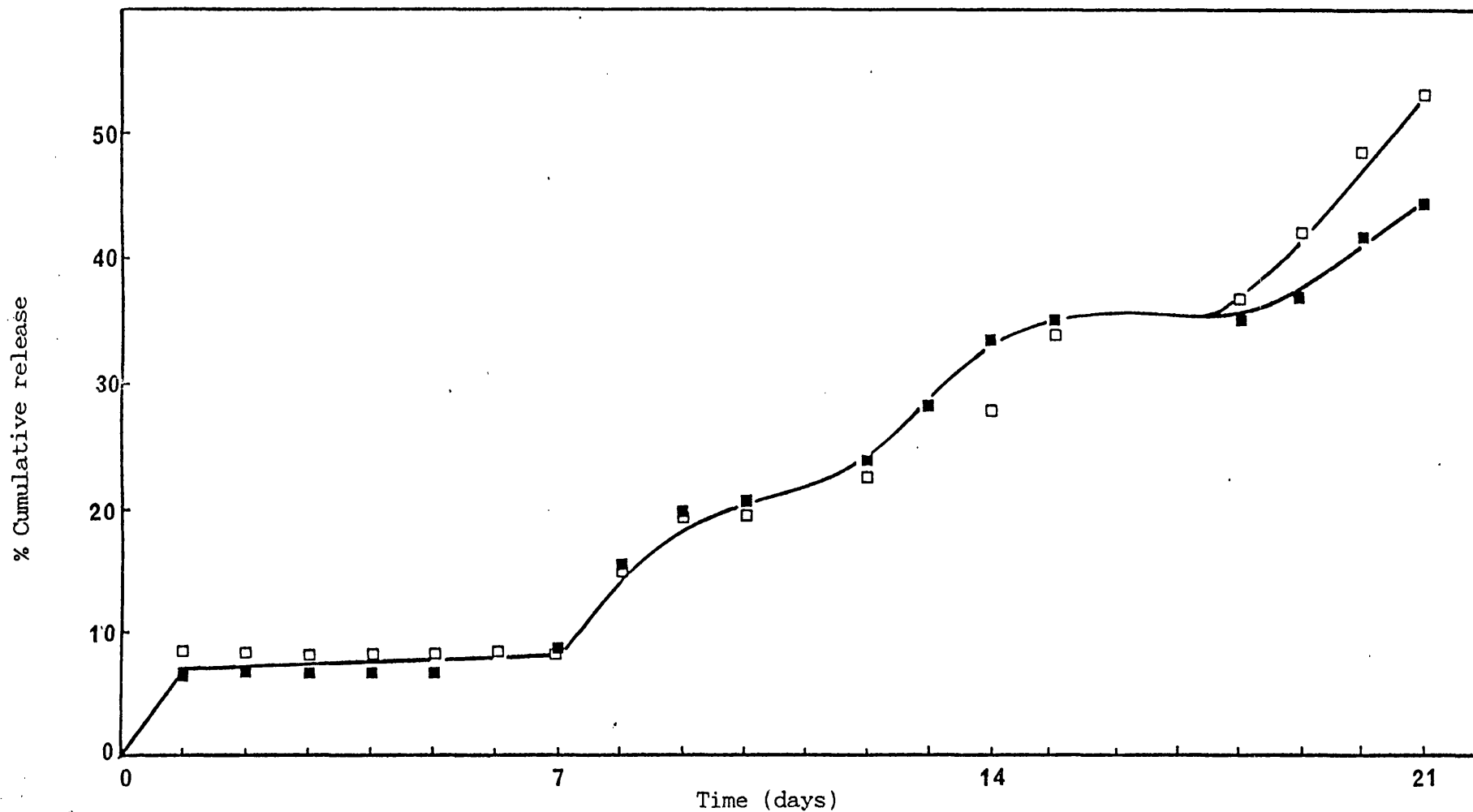


Figure 7.3. Cumulative percent release of 10% and 15% loaded inulin/PHB matrices versus time in days into (0.1M) NaOH release media pH 12.9 at temperature 37°C.

Points represent the mean release of 15 samples, S.D. <5% of the mean.

- 10% inulin loading.
- 15% inulin loading.

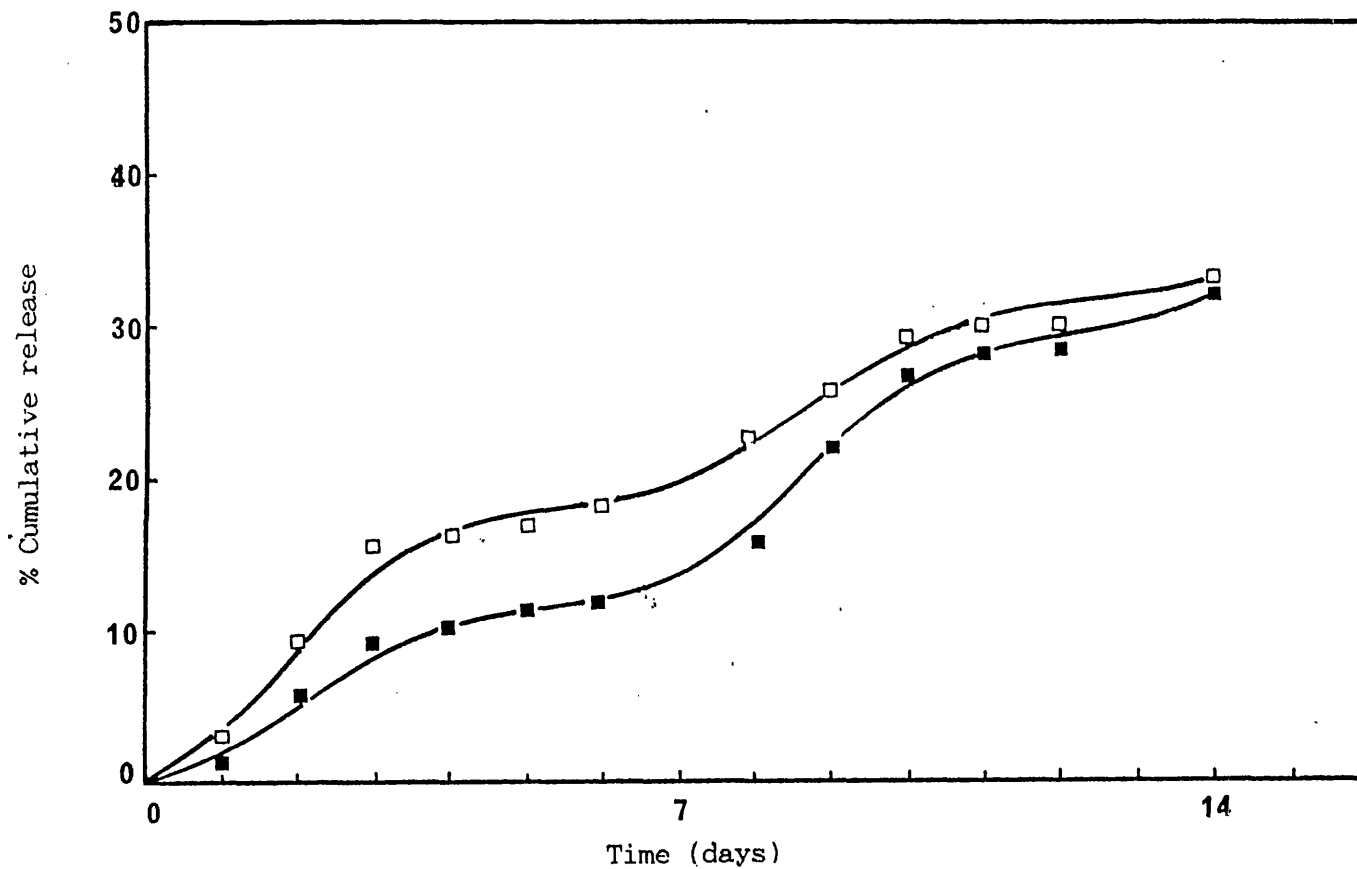


Figure 7.4. Cumulative percent release of 10%, and 15% loaded inulin/PHB matrices versus time in days into (0.1M) NaOH release media pH 12.9 at temperature 70°C.

Points represent the mean release of 15 samples, S.D. <5% of the mean.

■ 10% inulin loading.

□ 15% inulin loading.

Drug loading	Original weight (g +/- s.d.)	Weight after 14 day release period (g +/- s.d.)	Mean drug/polymer loss (mg)	Total % loss	% drug loss	Drug loss (mg)	Polymer loss (mg)	
1	10%	0.1149 +/-0.03	0.0858 +/-0.02	29	25%	34%	3.8	25.2
	15%	0.1098 +/-0.03	0.0947 +/-0.02	15	14%	28%	3.1	11.9
2	10%	0.1177 +/-0.01	0.0542 +/-0.01	63	54%	32%	3.8	59.7
	15%	0.1098 +/-0.01	0.0527 +/-0.02	57	52%	33%	3.6	53.5
3	10%	0.1205 +/-0.02	0.1102 +/-0.02	10	9%	16%	1.9	8.4
	15%	0.1055 +/-0.01	0.0929 +/-0.01	13	12%	23%	2.4	10.3
4	10%	0.1163 +/-0.01	0.1098 +/-0.01	7	6%	12%	1.4	5.1
	15%	0.1247 +/-0.01	0.1103 +/-0.00	14	12%	12%	1.5	12.9

Table 7.1. The effect of pH, drug loading and temperature on PHB (23K)/inulin melt extrudate release *in vitro*.

1 = pH 12.9; temperature 37°C

2 = pH 12.9; temperature 70°C

3 = pH 7.4; temperature 70°C

4 = pH 7.4; temperature 37°C

n = 15

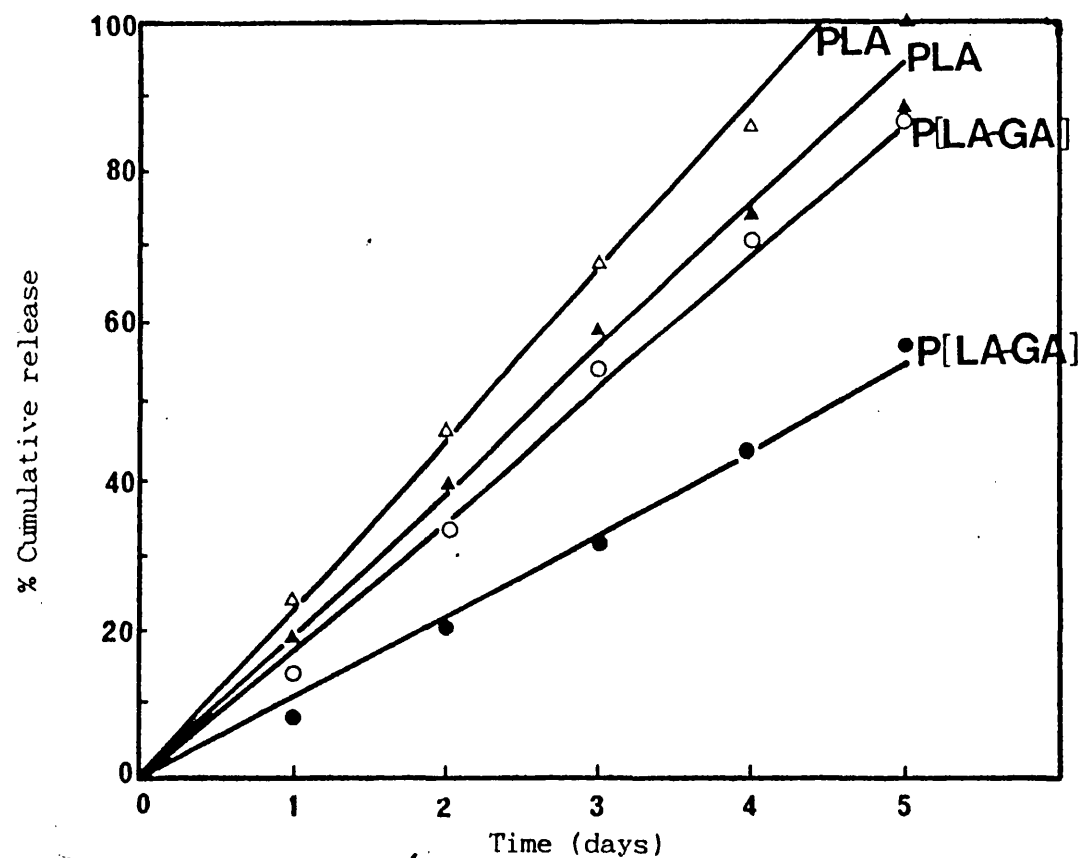


Figure 7.5. Cumulative percent release of 10% and 15% loaded inulin/PLA and inulin/P(LA-GA) 50:50 matrices versus time in days into (0.1M) NaOH release media, pH 12.9 at temperature 37°C.

Points represent the mean release of 15 samples, S.D. <5% of the mean.

▲,● 10% inulin loading.

△,○ 15% inulin loading.

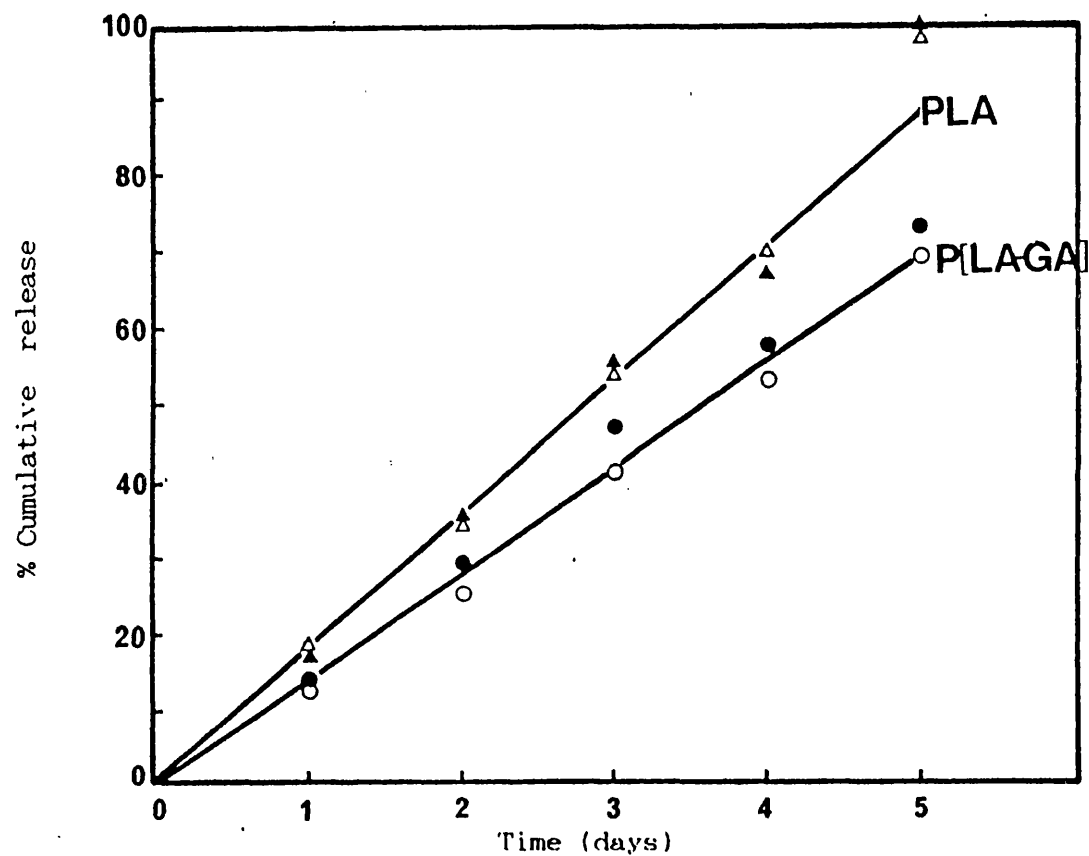


Figure 7.6. Cumulative percent release of 10% and 15% loaded inulin/PLA and inulin/P(LA-GA) 50:50 matrices versus time in days into (0.1M) NaOH release media, pH 12.9 at temperature 70°C. Points represent the mean release of 15 samples, S.D. <5% of the mean.  
 ○, △ 10% inulin loading.  
 ●, ▲ 15% inulin loading.

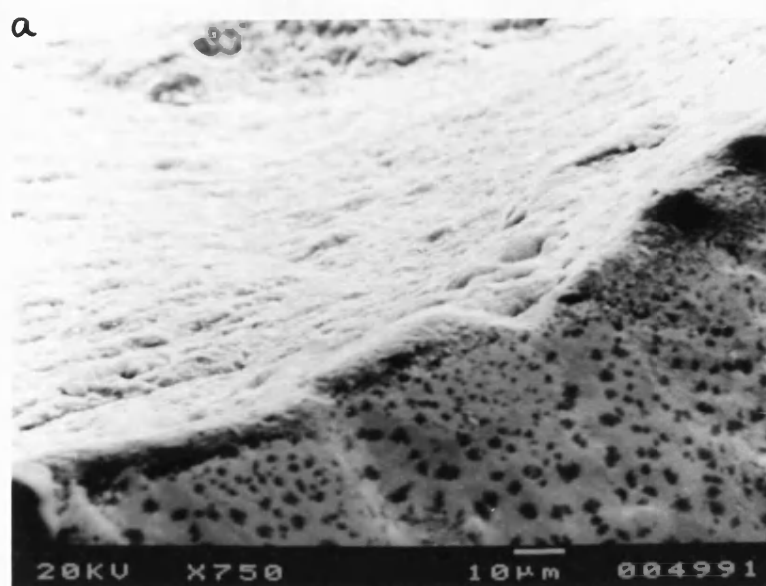


Figure 7.7. Scanning electron micrograph of inulin/PHB melt extrusion containing 15% by weight inulin, before release experiments.  
 a) Cut section of extrusion. x750 20KV.  
 b) Surface view. x500 20KV.

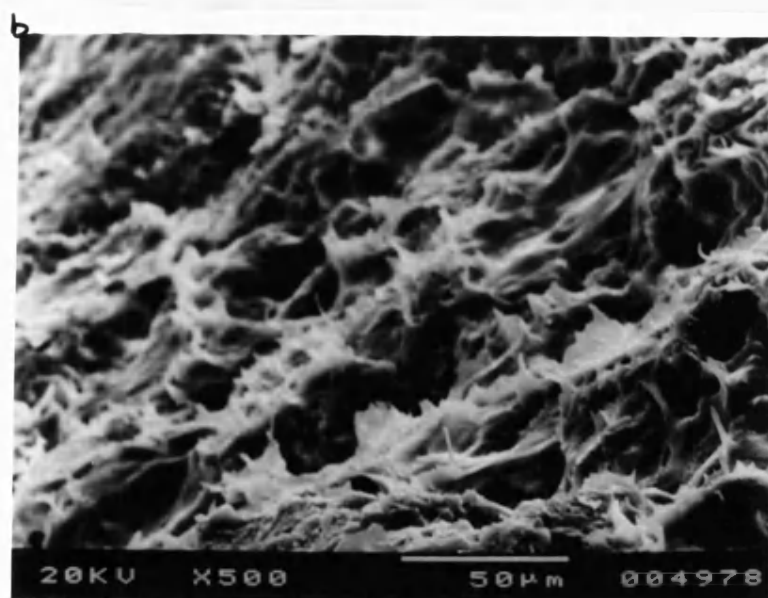


Figure 7.8. Scanning electron micrograph of inulin/ PHB melt extrusion containing 15% by weight inulin after 14d in (0.1M) NaOH, pH 12.9 and temperature 70°C.

a) Cut section of extrusion. x100 20KV

b) Surface view. x500 20KV



respectively. The release of both 10% and 15% inulin loaded PLA, P(LA-GA) matrix systems at both temperatures appeared to be linear with time. The rate of inulin release from PLA appeared to be greater than from the P(LA-GA) matrices at both drug loadings and both temperatures. Total degradation and drug release from these PLA, P(LA/GA) matrices had occurred by day 5.

At the end of the release study SEM suggested that PHB extrudates degraded by erosion as no internal pores were observed (figures 7.7-7.8).

#### 7.5.2. *In vivo* release .

Figure 7.9 compares the release kinetics of 10% loaded  $^{14}\text{C}$  inulin-P(LA-GA) matrices and 20% loaded  $^{14}\text{C}$  inulin-PHB matrices *in vivo* over a 28 day implantation period. Cumulative percent release of labeled inulin is plotted against time and each graph illustrates the releases kinetics from implanted extrudates for individual animals. The release rate of  $^{14}\text{C}$  inulin from PHB matrices was between 3-5% over the 28 day period. The P(LA-GA) systems however released between 25-40% of the total incorporated drug. P(LA-GA) systems exhibited two phases of release. The initial phase of release was slow and steady over the first 10 days. In the second phase of release a burst of release occurred over two days which then became steady, giving near zero order release kinetics over 12-28 days. The PHB loaded systems released inulin steadily over 28 days. At the end of the studied release period the implants were removed, dried and weighed. The PHB/drug matrices had lost very little weight, (~4%) whereas the P(GA-LA) matrices lost ~20% of their initial weight. SEM revealed no porosity within the PHB/drug matrix and very little surface pitting after the implantation time. (figure 7.10). The

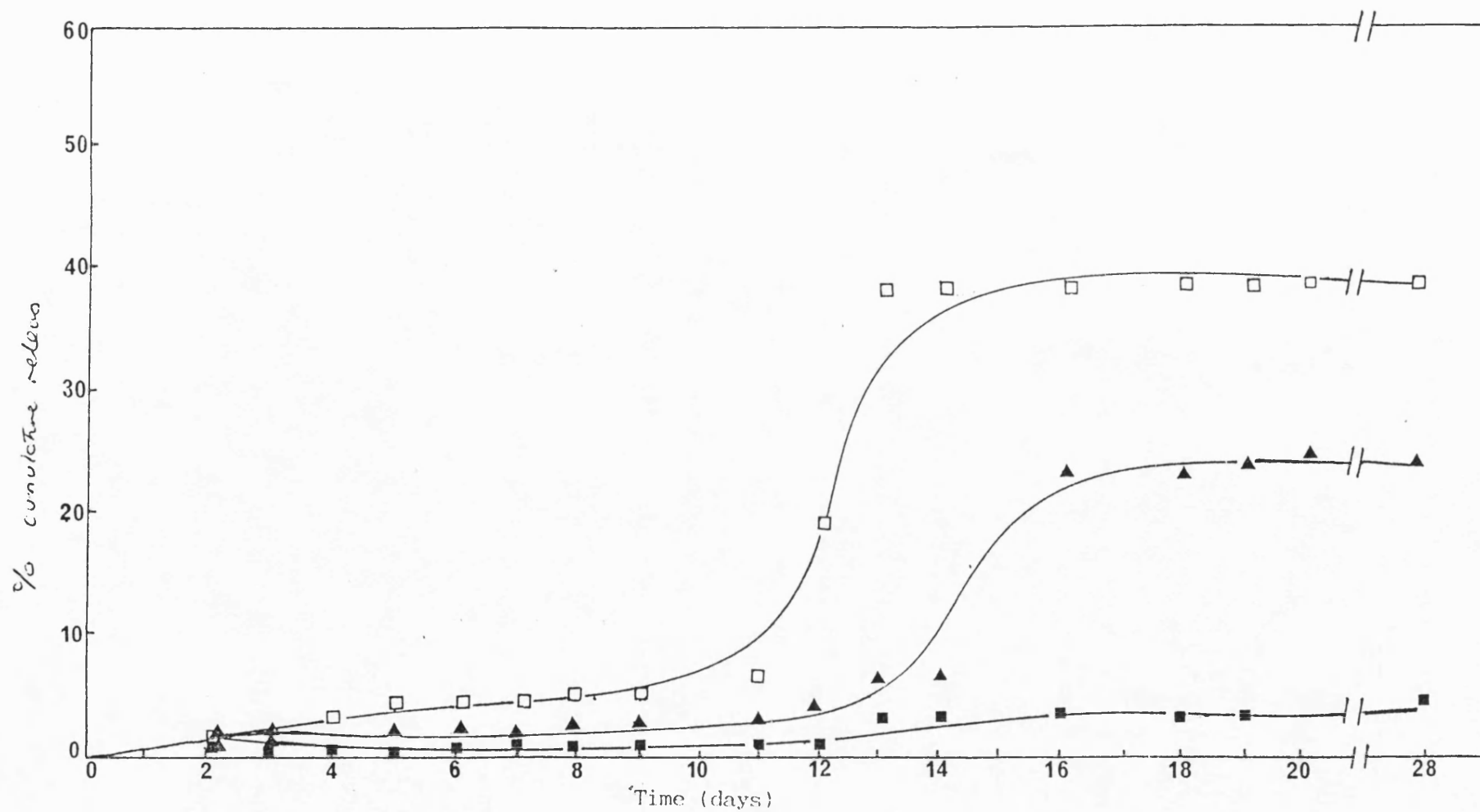


Figure 7.9. The release kinetics of  $^{14}\text{C}$  inulin from PHB and P(GA-LA) extrusions *in vivo*.

- 20% drug loading in PHB (n=3; S.D <5% of mean).
- 10% drug loading in P(GA-LA) extrusions (n = 1).
- ▲ " " " " " " " " " " " " " " " "

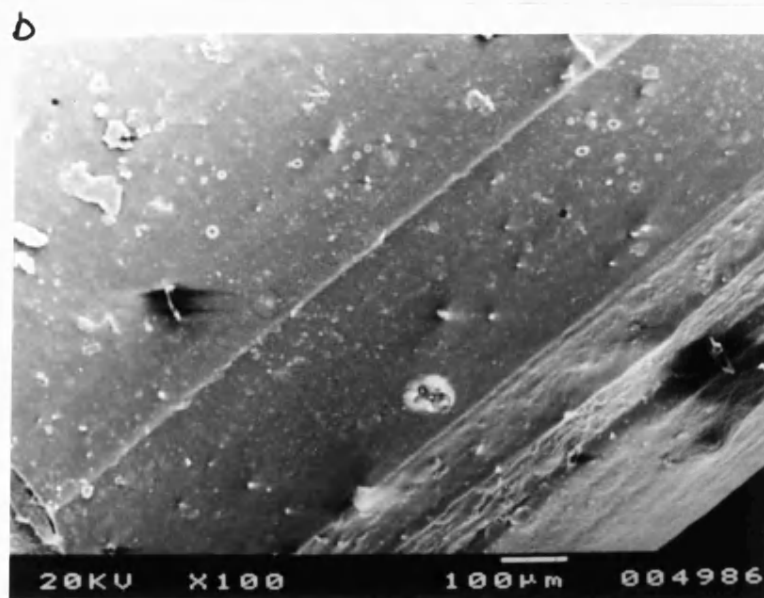
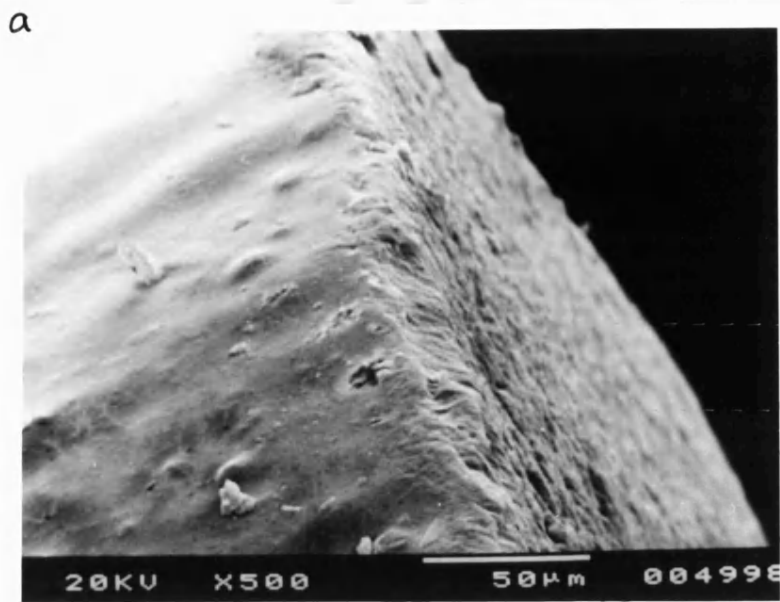


Figure 7.10. Scanning electron micrograph of inulin/PHB melt extrusion containing 20% by weight inulin, after 28d implantation into rat gluteal muscle.

a) Cut section of extrusion. x500 20KV.  
b) Surface view. x100 20KV.

P(LA-GA) systems were porous and pitted at the surface which was probably explained by drug release via diffusion and homogenous (bulk) erosion (figure 7.11).

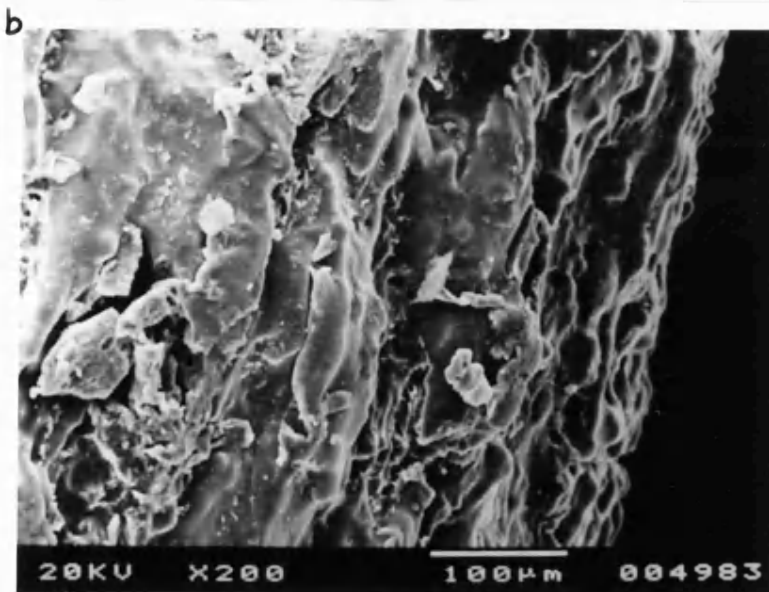
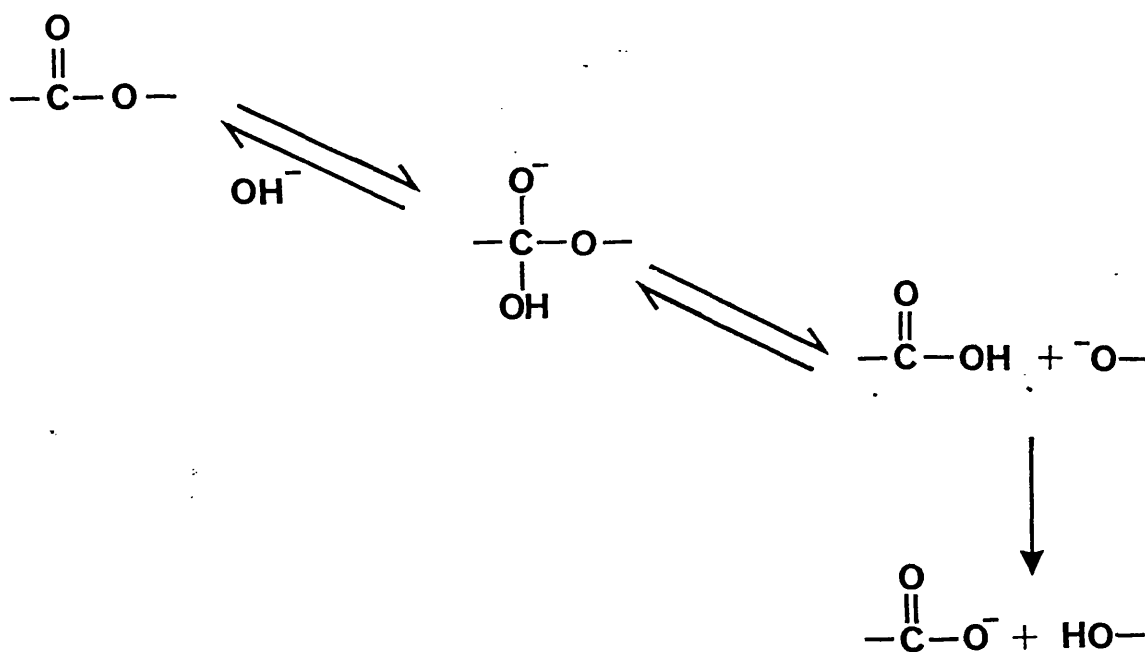


Figure 7.11. Scanning electron micrograph of inulin/P(LA-GA) 50:50 melt extrusion containing 10% by weight inulin, after 28d implantation into rat gluteal muscle.

a) Cut section. x100 20KV  
b) Surface view. x200 20KV

### 7.6. Discussion.

*In vitro* degradation of PHB and subsequent inulin release has shown the influence of temperature and pH on the rate of matrix buffer hydrolysis and drug release. The rate of drug release and extent of overall polymer degradation in aqueous buffer solutions, one at physiological pH (7.4) and one in alkali conditions (pH 12.9) increased with temperature in accordance with the proposed ester hydrolysis mechanism (equation 1). The enhancement of overall polymer degradation and drug release in alkaline hydrolytic medium was also evident. This was expected since ester hydrolysis is often base catalysed.



Degradation of polyesters in basic solutions. (Schnabel, 1981)

A change in drug loading between 10 and 15%w/w did not appear to influence greatly the overall drug release from PHB matrices or

the overall extent of polymer degradation. This suggested that the polymer degraded at the surface and that drug was released only when exposed by polymer degradation. SEM micrographs revealed that the surface of PHB extrudates became increasingly pitted and rough but the internal structure remained solid during the time spent in the aqueous buffer media. At physiological pH and temperature the release kinetics of inulin from the extrudates was closely related to loss of polymer but as the temperature and pH was increased to accelerated degradative conditions, 70°C and pH 12.9 (Majid, 1988), drug release became less predictable. Surface erosion of PHB and P(HB-HV) in the initial stages of degradation was measured by Holland *et al*, 1987. Combined dry and wet gravimetric results together with surface energy and goniophotometric measurements showed that in the initial stages of degradation little change in the bulk or mass of the sample was detected and an increase in the concentration of hydroxyl and carboxyl groups at the surface as a consequence of ester hydrolysis at the polymer-water interface was evident. As degradation proceeded bulk erosional processes became operative such that the matrix became more porous allowing an increasing loss of higher molecular weight degradation products. The period of degradation in aqueous media (pH 7.4) at a temperature of 70°C used by these researchers was typically 70 days. The process of bulk degradation was not apparent until day 28 after which substantial mass loss equivocal to bulk hydrolysis occurred. As the degradative period used in the preliminary drug release experiments presented in this chapter was only 14 days it may be that the initial surface erosion observed would later have been followed by bulk hydrolysis. This would explain the drug release kinetics that were observed in the experiments where accelerating conditions were used. If drug diffusion through the bulk matrix

becomes important one would expect the Higuchi model to apply (equation 2).

The Higuchi model predicts that the cumulative release of drug from matrices would be linear with the square root of time if the following assumptions are met (Brown *et al*, 1986).

- 1) initial drug loading is greater than the solubility limit of the drug,
- 2) the polymer portion of the matrix is not miscible with the release media,
- 3) the release medium provides perfect sink conditions
- 4) pseudo steady-state conditions exist to allow the sustained release of drug from the matrix.

$$Q = (DC_s / \tau)(2A - C_s)t^{1/2} \text{ ----- (2)}$$

where Q = quantity of drug released per unit area at time t(mg/cm<sup>2</sup>); D = effective diffusion coefficient (cm<sup>2</sup>/s); C = inulin solubility in the release media (mg/ml);  $\phi$  = porosity of the inulin-polymer matrix;  $\tau$  = tortuosity of the matrix; A = weight loading of inulin in the volume of the matrix; t= time (s).

The rate of drug diffusion under these conditions is therefore dependent on the size and tortuosity of the pores. Fabrication variables that affect the structure of these pores are drug particle size (Langer and Folkman, 1980; Brown *et al*, 1983, 1986), drug loading (Suzuki and Price, 1985), and the matrix preparation technique (Rhine *et al*, 1980).

The formulation of the polymer/drug matrix can also influence rate of polymer degradation and therefore drug release kinetics. Holland *et al* have also shown that degradation rates occur in the following rank order: compressed tablets < solvent cast films < melt



pressed discs < injection moulded pieces. These differences correlated with the difference in degrees of crystallinity as measured using a differential scanning calorimeter. High crystallinity was associated with high resistance to hydrolytic attack. The drug release of inulin was therefore likely to have been retarded by the melt extrudate formulation. This was apparent in *in vivo* release studies where the release of 20% drug loaded inulin/PHB melt extrusions was negligible over 3-28 days. P(LA-GA) (50:50) melt extrusions with 10% drug loading however released 25% of the inulin over 28 days, although release was uncontrolled. The difference in release kinetics *in vivo* could be attributable to different degrees of crystallinity in the two melt extruded polymers.

Drug release studies from PHB have been carried out by Korsatko *et al*, 1983a, 1983b. PHB matrix tablets as sustained release formulations were prepared by compressing homogenous mixtures of PHB and 7-hydroxyethyltheophylline (HET). The tablets were 2.00mm in thickness and 6.00mm in diameter. A variety of drug loadings were studied ranging between 5% and 80%. *In vitro* release studies were performed in 0.9% NaCl at 37°C. At drug loadings below 30% w/w release took place over a period of 50 days whereas with drug loadings of 60-80% w/w release was completed within 24 hours. Release of drug occurred initially from the surface and then proceeded by diffusion through aqueous pores formed by water penetration of the matrix of the tablet. The degradation of PHB had no significant effect on the release of HET *in vitro*.

Bissery *et al*, (1984) prepared PHB microspheres containing an anticancer drug and *in vitro* drug release was studied. In buffer of pH 7.2 at 37°C more than 90% of the loaded drug (7.4% w/w) was released in 10 hours. However release rate of the drug from P-dl-LA

microspheres prepared by the same method, showed that release was sustained over a longer period i.e. 60% of drug released in 90 hours. The authors were unable to explain the results.

Drug release from PHB microspheres has also been studied by Juni *et al* (1986) and Brophy and Deasey (1986). Release rates were dependent on a number of variables including drug loadings, particulate size, solvent evaporation process, molecular weight and model drug used. Geometry of the matrix device is also another important variable often used to control the release kinetics of drugs from the matrix (Fessai *et al*, 1979; Pitt *et al*, 1979). Hofenberg (1976), presented equations describing the idealised kinetics for erodible slabs, cylinders and spheres, indicating that constant delivery is provided only by the slab geometry. The cylindrical drug/polymer matrices used in the *in vitro* and *in vivo* studies presented in this chapter would according to this theory be expected to initially deliver the drug at a constant rate, but as surface erosion occurred, the drug release would begin to decrease as the surface area available for release decreased. This however was not observed possibly due to the time span of the experiments. The nature of the PHB-drug matrices therefore clearly varied and further study in our laboratories needs to be done before conclusive evidence of the mechanism of polymer degradation and therefore drug release can be obtained.

The rate of PLA and P(LA-GA) degradation in contrast to PHB was significantly greater ( $p < 0.001$ ) and drug release appeared to follow Higuchi's mathematical model, i.e. cumulative release is linear with square root of time (figures 7.5-7.6). Accelerated conditions of degradation were examined and only one copolymer composition (50:50) was examined.

The effect of copolymer composition on the release of a hormonal steroid, was studied by Sharon *et al* (1984). The release of the steroid was directly related to the susceptibility of each polymer to degradation. Copolymers and homopolymers of l-LA, dl-LA were used. The rank order of polymers with increasing rate of biodegradation was P(75% l-LA/25%GA), P(90% dl-LA/10%GA), P(100% dl-LA), P(50% dl-LA/50%GA) and P(100% l-LA). Two polymers P(100%dl-LA) and P(50%dl-LA/50%GA), were selected for further release studies of the steroid since they appeared to have potential for development of a delivery system with a lifetime of 6 months to 1 year.

The controlled release of peptides, typically LHRH analogues, from P(dl-LA/GA) systems have been studied extensively (Sanders *et al*, 1986; Hutchinson and Furr, 1987; Kaetsu *et al*, 1989; Asano *et al*, 1989; Ogawa *et al*, 1989). Sanders *et al* (1984) prepared microspheres by a phase separation method. The peptide in aqueous solution was mixed with a solution of P(50%dl-LA/50%GA) in dichloromethane. The release of the peptide from these microspheres was found to be triphasic. The primary phase involved diffusion of free drug from the surface of the microsphere, followed by a secondary phase where the release rate was too low to be therapeutically effective, followed by a tertiary release phase of the peptide through bulk erosion of the polymer.

Matrices can be formulated to give biphasic releases avoiding the slow secondary phase release (Hutchinson and Furr, 1985). This was achieved by use of low molecular weight polymer and a higher loading of drug. High molecular weight P(d-LA) (intrinsic viscosity = 1.02 dl/g) resulted in extension of the secondary phase whereas an implant prepared using a low molecular weight polymer (intrinsic

viscosity =0.36dl/g) exhibited no secondary phase of release. Molecular weight was also seen to affect the melt extrudate fabrication process of P(HB-HV). The average molecular weight was 760K compared to 23K, 28K and 56K of PHB, PLA and P(LA-GA) respectively. At this high molecular weight, even at elevated temperatures and extended extrusion times, the viscosity of the melt was too large to be extruded and if peptides had been used thermal degradation would certainly have occurred.

Research in the area of biodegradable polymers as controlled drug delivery devices is a continually growing field. Biodegradable polymers which are not only biocompatible but also ideally release macromolecules in the body to give an optimal therapeutic effect have still to be found. Of the biodegradable, parenteral, controlled-release delivery systems, only one implant (Hutchinson and Furr, 1987) and two injectable microsphere products have been introduced, each of which use lactide polymer or lactide/glycolide copolymer as the excipient to control the release of the drug. With a single administration the implant (Zoladex,ICI) and one of the microsphere products (Decapeptyl; Ipsen Biotech) deliver luteinizing hormone-releasing hormone agonists for one month for the treatment of prostate cancer. The second microsphere product delivers bromocriptine for a one month period (Parlodel, Sandoz).

It has taken some time for the first parenteral controlled-release products to appear because of their complexity as well as the need to solve many of the practical problems of manufacture and marketing. Polymers and copolymers of lactic and glycolic acid have been at the fore front of this research because of their well established and reported biocompatibility. However other biodegradable polymer formulations, in particular the polyester PHB

and its copolymers under research at Bath University and the polyorthoesters, currently under reseach by Heller and his colleagues, are now being investigated as controlled peptide delivery systems because of their mechanism of degradation (i.e. surface erosion giving controllable drug release) and their biocompatibility. Extensive degradation studies and more succinct drug release studies need to be performed both *in vivo* and *in vitro*, before any real conclusions can be drawn from these initial release studies.

## CHAPTER 8.

### 8.1. Concluding Discussion.

The importance of tissue reactions to polymer implantation has been addressed by many research scientists concerned with biocompatibility (Bischoff, 1972; Rigdon, 1974; Coleman *et al*, 1974; Leong *et al*, 1986; Visscher *et al*, 1986; Sevastjanova *et al*, 1987; Tamargo *et al*, 1989) and the techniques used to measure such tissue reactions have been the subject of a number of reviews (Salthouse, 1976; Rae, 1986; Meachim and Pedley; 1986). Over the years the selection of biocompatible polymers has been achieved empirically, testing materials by implantation into animals.

The use of animal models, such as the rat and rabbit, in the initial stages of biocompatibility testing is expensive both in time and resources and therefore some form of *in vitro* pre-screening is desirable to ensure efficient usage of subsequent animal testing. Tissue culture methods in particular have been employed in the evaluation of the toxic potential of polymers by a number of workers, early work carried out notably by Guess and Autian (1964), Grasso (1973) and Wilsnack (1973). At present there is no clear distinction between the terms biocompatibility and toxicity (Styles, 1986) and it is therefore assumed that the degree of toxicity measured *in vitro* may be used to predict an incompatibility *in vivo*.

Chapter 3 described *in vitro* cell culture experiments to determine whether *in vivo* biocompatibility tests could be justified for the polymers PHB, P(HB-HV) and their degradation products. Experimental evidence showed that PHB, P(HB-HV) and low concentrations of their monomers were not cytotoxic to CHO-K1 cells. However at higher concentrations of sodium HB and HV moderate to severe cytotoxicity occurred. This cytotoxicity was believed to be due

to an osmotic effect within stagnant culture conditions since sodium HB and HV were no more toxic than sodium chloride or sucrose on a molar basis. The local concentration of degradation products would never reach such high levels *in vivo* due a) rapid diffusion away from the implant site and b) the slow rate of degradation of the implanted polymers. A cell culture test system, while of obvious benefit, can lead to incorrect conclusions due to the closed nature of the culture as in the cases of HB and HV. It is also possible that an underestimation of toxicity may be observed, since some substances are inactive in their native form but may be transformed into a toxic form by action of the liver microsomal enzyme system, the potential for such a transformation being absent in tissue culture (Styles, 1986). Another disadvantage of tissue culture is that it can only test for acute toxicity as only short culture periods can be maintained. Therefore if a polymer is judged to be compatible in cell culture tests then *in vivo* testing will have to be performed not only to substantiate *in vitro* evidence of biocompatibility but also to establish the biocompatibility of materials for long term implantation. The *in vitro* cell culture tests represented a first step in assessing the biocompatibility of PHB, P(HB-HV) and their monomers. However cell culture was not able to model the haemodynamic, haematological and reticulo-endothelial compensatory mechanisms found *in vivo*.

*In vivo* biocompatibility tests were designed to determine the tissue reactions to implanted PHB, P(HB-HV) and their monomers. With regard to the proposed use of these materials as drug delivery systems, the protocol concentrated on assessment of inflammatory reactions in soft tissue and blood compatibility. The overall inflammatory response to the polymers and monomers *in vivo* was

assessed, the results of which are reported in chapter 6. Individual aspects of the inflammatory response were examined *in vitro*, the results of which are documented in chapters 3-5. The purpose of this general discussion is to interrelate data reported in chapters 3-6.

Implantation or injection of any material into soft tissue will initiate an inflammatory response if only that resulting from the surgical trauma induced. Injection or implantation causes acute tissue damage and vascular beds are disturbed. One of the first results of such damage is the leakage of plasma proteins, erythrocytes, white blood cells and platelets (Bult and Herman, 1985; Ito *et al*, 1989) to the site of trauma to aid tissue repair and wound healing. Tissue damage also leads to the release of inflammatory mediators which increase vascular permeability and chemotactically attract cells of the immune response, primarily PMNs and macrophages, (Behling and Spector, 1986; Marchant *et al.*, 1983,1986; Callis *et al*, 1988; Williams *et al*, 1989). Exacerbation or resolution of this inflammatory response is then dependent on

- 1) interaction of the polymers with plasma proteins
- 2) macrophage adherence/ activation/ phagocytosis
- 3) fibrin formation
- 4) tissue encapsulation

A summary of the biochemical and cellular events, that can occur following implantation of a material into soft tissue in the short term (acute phase), and long term (chronic phase), are presented in figures 8.1 and 8.2 respectively. The magnitude and duration of the inflammatory response is often related to the interaction of inflammatory cells, PMNs and macrophages, with the biomaterial. These cells are chemotactically attracted to the inflammatory focus initially by histamine, PGE<sub>2</sub> and lysosomal



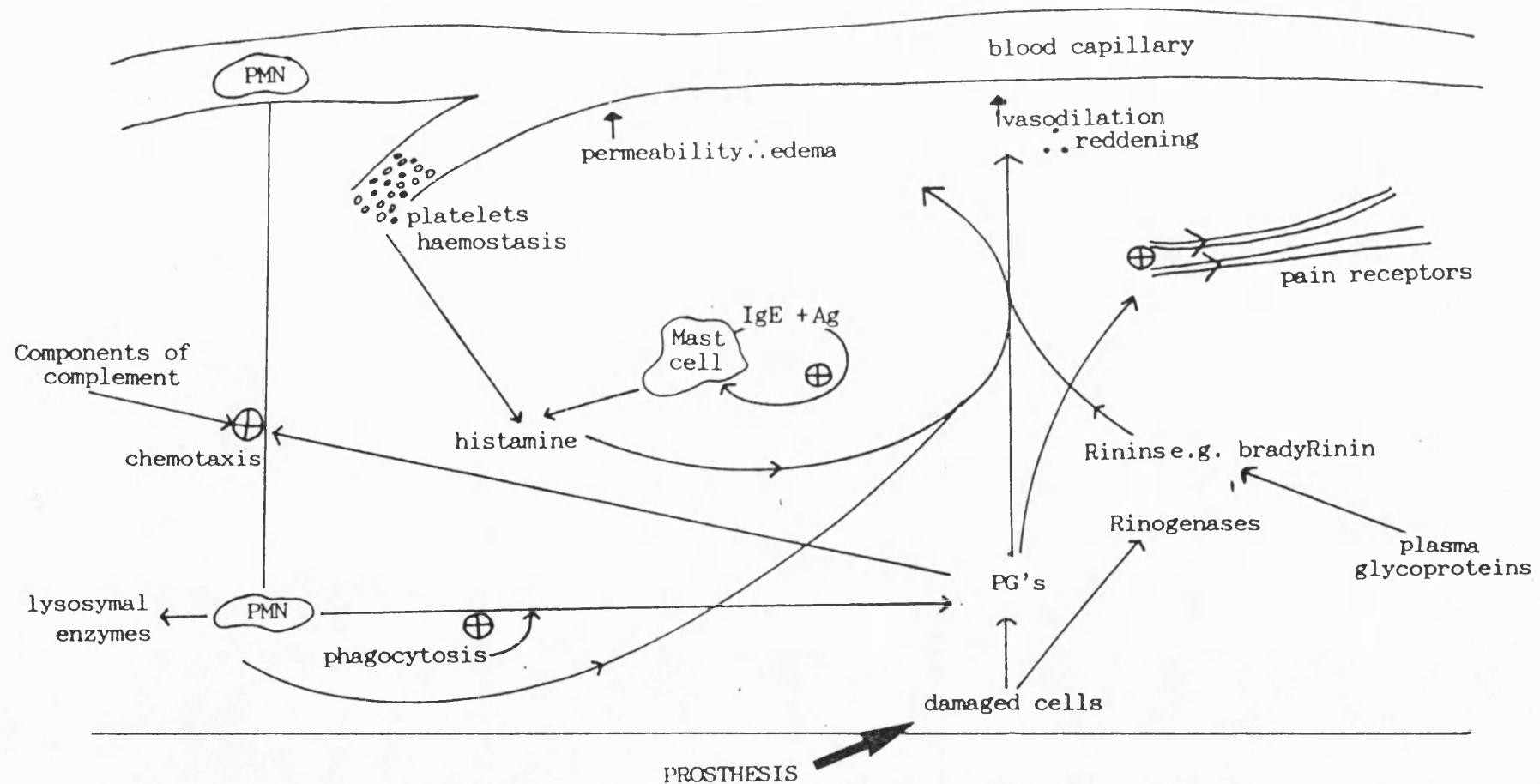


Figure 8.1 Summary of the biochemical events of the acute phase of inflammation to an implanted prosthesis. ⊕ indicates a promoting or activating effect on a particular process; ↑ indicates an increase effect; PMN, polymorphonuclear leucocyte; PG, prostaglandins, IgE, immunoglobulin IgE; Ag, antigen; and ?, unconfirmed effects.  
(Reproduced from T. Rae, *Fundamental Aspects of Biocompatibility*; Ed. D.F. Williams, 1981.)



hydrolases (Rae, 1981), all of which are released by damaged tissue cells. Histamine and PGE<sub>2</sub> increase vascular permeability (Baggiolini, 1985) leading to an increase in plasma exudation, and in the initial stages of the inflammatory reaction, 1-3 days, PMNs extravate into the area of inflammation. Differential increases in vascular permeability due to release of chemical mediators from the inflammatory cells depend on the number of inflammatory cell polymer interactions which then often relate to the extent of tissue damage observed during implantation of the different polymers (Ziats *et al*, 1988). Vascular permeability studies *in vivo*, reported in chapter 6, showed that injection of PHB and P(HB-HV) into the gluteal muscle of male Wistar rats did not significantly ( $p>0.1$ ) increase the permeability of the vasculature. A small increase in vascular permeability was observed which was considered to be attributable to the trauma of injection. Although the effects on vascular permeability *in vivo* were measured only after 2 and 4 hours injection, the little effect on increase of vascular permeability correlated well with a mild and transient inflammatory response, observed macroscopically over the first 3 days following polymer implantation.

The increase in vascular permeability seen after implantation of polymers compared to that measured following the insertion of a needle (sham injection) was insignificant. This correlated with data on PGE<sub>2</sub> production and release by cultured macrophages *in vitro*, reported in chapter 4. It is generally accepted that PGE<sub>2</sub> biosynthesis is involved in the development of an acute inflammatory response in several models of acute inflammation (Ferreira and Vane, 1979; Bonta and Parnham, 1978). PGE<sub>2</sub> exerts its pro-inflammatory activity by dilatation of the resistance vessels, thereby drastically

increasing blood flow through the affected tissue. However the biosynthesis of vasodilating prostaglandins is a rate-limiting factor in the fluid exudation since simultaneous application of PGE<sub>2</sub> and PGE<sub>1</sub> together with inflammatory mediators like carrageenan and zymosan greatly potentiates the early exudation (Williams, 1979). In studies with PHB and P(HB-HV) an initial significant production and release of PGE<sub>2</sub> by macrophages in culture during the first hour of contact was evident. This was believed to be a result of macrophage activation. After 2 hours, PGE<sub>2</sub> production and release decreased due to either an acute release of the prostaglandin upon polymer contact causing depletion of PGE<sub>2</sub> and a latent period during which the prostaglandin is resynthesised, or cellular down regulation of PGE<sub>2</sub> release, or the production of chemical inhibitors of prostaglandin production. The measured small effect on the permeability of the vasculature around the area of implantation of the polymers in the initial stages of the inflammatory response, and the subsequent decrease of PGE<sub>2</sub> production and release from activated macrophages in the later stages of the inflammatory response would predispose to an inflammatory response which would likely be quickly resolved and not exacerbated.

PGE<sub>2</sub> is produced and released only on activation of the inflammatory cells (Lasser, 1980;). However adhesion of these cells to the biomaterial is required before activation of the cell occurs (Rabinovitch, 1970). Therefore the presence of these cells in the inflammatory exudate around an implanted polymer does not necessarily correlate with subsequent activation. Adhesion to the polymer surface by these cells is thought to occur to an adsorbed protein layer.

One of the first events that occurs following implantation of a polymer into soft tissue, is the adsorption of plasma proteins onto

the polymer surface. Proteins which may adsorb to the surfaces of biomedical polymers are widespread ranging from albumin, thrombin, fibrinogen, to extracellular matrix proteins such as fibronectin and IgG immunoglobulins (Vroman *et al*, 1980; van Wachem *et al*, 1987; Pitt *et al*, 1988). Then as a function of the plasma protein adsorbed, inflammatory cells such as PMNs and macrophages may adhere. PMNs and macrophages both have receptors for the Fc portion of gammaglobulin which will cause cell adhesion if a surface adsorbs gammaglobulin (Rae, 1981; Anderson and Miller, 1984). Activation can then occur via a number of interfacial processes, the mechanisms of which are yet to be defined. These proteins therefore act as opsonins, interacting with different populations of cells to induce possible activation (Patarroyo *et al*, 1988; Bonfield *et al*, 1989). In particular, interactions of the opsonins with the receptors that exist on PMNs and the macrophages can cause the cell to phagocytose (Pommier *et al*, 1983) or secrete products to the surrounding environment (Ziats *et al*, 1988) which may resolve or exacerbate the inflammatory response. Studies in a number of laboratories suggest that biomedical polymers depending on their structure and composition, preferentially and differentially adsorb certain plasma proteins (Marchant *et al*, 1986; Lee *et al*, 1989; McCoy *et al*, 1989). These properties may influence subsequent cellular attraction to the site of polymer implantation as well as cellular adhesion followed by activation. This in turn induces the release of more inflammatory mediators into the implantation area, chemotactically attracting more inflammatory cells to the area.

Research by Bonfield *et al* (1989), has linked the adsorption of certain plasma proteins to the activation of macrophages and subsequent release of another important inflammatory mediator,

interleukin 1 (IL1). These workers found that when Biomer, PDMS, PEE, Dacron and PTFE adsorbed IgG and fibrinogen, the resulting production of IL1 was retarded in comparison with the protein coated polystyrene controls. This implied that the interaction between IgG and fibrinogen with polymers caused a decrease in cellular activation. Conversely if the polymer remained uncoated or was pre-coated with albumin no suppression of IL1 production and release was observed. The suppression observed in these studies could be the result of a variety of factors including protein-cell interactions, induction of IL1 inhibitors or direct cellular down-regulation, which in turn could play a part in the the inhibition of IL1 secretion from activated monocytes. This type of response was also believed to occur with PHB and P(HB-HV) since both uncoated polymers initially stimulated the macrophage to produce another important inflammatory mediator, PGE<sub>2</sub>. This could have been explained by direct cellular down-regulation mediated by the biomedical polymer itself, or other cellular down-regulation mechanisms. However in view of the protein adsorption data presented in chapter 5, where albumin and gammaglobulin equally competed with each other for adsorption to the surface of PHB, then in whole blood where the concentration of albumin is greater it would be predicted that macrophages would not adhere well to PHB polymer surfaces since albumin does not contain the necessary recognition sites for macrophage binding. Therefore activation of macrophages which led to a burst in PGE<sub>2</sub> production and release *in vitro* in the presence of uncoated PHB, would not occur *in vivo*. However, P(HB-HV) preferentially adsorbed IgG, which include the receptors for macrophage attachment. Thus it could be predicted that IgG-coated P(HB-HV) could activate macrophages leading to the release of PGE<sub>2</sub>, which would increase vascular permeability and

destruction of the tissue cells leading to exacerbation of the inflammatory response. This is contradicted by the work of Bonfield *et al.* who showed that biomedical polymers pre-coated with IgG, appeared either to suppress or have no effect on the production of the inflammatory mediator IL1. This suggests either that protein cell interactions on the surface of the biomedical polymers inhibited mediator production by unknown cellular down-regulation mechanisms.

Marchant *et al* (1983) used a cage implant system to quantitate the humoral and cellular components of the inflammatory response to the biodegradable hydrogel, poly(2-hydroxyl-ethyl-glutamine), (PHEG). They illustrated the importance of cell-surface adhesion to exocytosis of inflammatory mediators into the site of inflammation. Exocytosis of mediators was insignificant in the presence of the chemotactic stimulus when the cells were non-adherent. The authors concluded that a nonadhesive pathway to exocytosis by PMN's does not play a major role in providing extra-cellular enzymes in the exudate. Utilizing *in vitro* studies, Henson (1980) has shown that neutrophils adherent to complement-coated and immunoglobulin-coated nonphagocytosable surfaces may release enzymes by direct extrusion of the granules to the exterior of the cell. Henson also observed that the amount of enzyme released during phagocytosis was dependent on the size of the polymer particle, with larger particles inducing greater amounts of enzyme release. He also suggested that the specific mode of cell activation caused by a normal inflammatory response is dependent on the size of the implant and that a material in powder or particulate form, suitable for phagocytosis, may provoke a different degree of response than the same material in a non-phagocytosable form, such as a film. This may have further implications regarding inflammatory cell function, the extent and

duration of the different stages of the inflammatory response and the mechanisms by which inflammatory cells respond to a foreign body stimulus. Furthermore, the availability of the two pathways for expression of cell activation, phagocytosis versus exocytosis, may result in problems of interpretation of cell culture studies where particulates are used.

Following PMN migration and phagocytosis/exocytosis by these cells, enzymes are released into the surrounding area and macrophages, associated with the later (chronic) stages of inflammation are chemotactically attracted to this area. Macrophage accumulation following intraperitoneal injection of polymers is documented in chapter 4. The extent of activation of these cells by PHB and P(HB-HV) was assessed *in vitro* by assay of PGE<sub>2</sub> (see chapter 4) and *in vivo* by assay of acid phosphatase (chapter 6). Both PMNs and macrophages contain intracellular acid phosphatase (Salthouse and Matlaga, 1981; Marchant *et al.*, 1986) the greatest amount of which is found in the macrophage. However the measured acid phosphatase activity could reflect both the acute and chronic phases of inflammation. Examination of data in chapters 4 and 6 reveals that there does not appear to be a direct correlation between PMN concentrations and the acid phosphatase levels. In contrast a correlation was observed with the alkaline phosphatase levels. At later time points the contribution from other leukocytes namely macrophages undoubtedly became more important. A direct correlation is seen between the macrophage accumulation data documented in chapter 4 and the acid phosphatase activity documented in chapter 6 over a 40 day implantation period. Peak acid phosphatase activity occurred at approximately the same time points as maximum macrophage accumulation. Both these parameters were greatest, after injection



of PHB microspheres, on days 4-8. Increases were observed after injection of P(HB-HV) microspheres but they were not as large as those observed with the homopolymer. The temporary increases could have been caused by attempted phagocytosis of the polymer microspheres resulting in exocytosis of enzymes and inflammatory mediators such as PGE<sub>2</sub>. However all results relating to aspects of the inflammatory response indicated that the duration of the inflammatory response was acute, that exacerbation did not occur and that phagocytosis did not occur. Injection of PHB and P(HB-HV) microspheres and even implantation of polymer films did not adversely affect the soft tissue into which they were implanted. It can therefore be concluded that the polymers themselves are inert and well tolerated. The histological assessment of the implantation of these polymers in soft tissue also correlated well with the results of vascular permeability, enzyme histochemistry and macrophage activation.

It was conceivable that biodegradation of these polymers could initiate a series of acute inflammatory conditions within the tissue in response to the degradation products which could eventually lead to necrosis of the tissue. Therefore each aspect of the inflammatory response was assessed with respect to high concentrations of the degradative products. It was assumed that effects due to release of soluble materials should only be attributable to the monomers as no additives, stabilisers or catalysts, used in synthetic production of biopolymers, are present in these bacterial biosynthesised polymers. The assessment of the inflammatory response to doses of the two monomers, far exceeding any expected concentration which would result from degradation of the polymers *in vivo*, indicated that even at high doses the soft tissue

inflammatory response was transient only and no necrosis was observed. *In vitro* observations of the inflammatory response to high doses of monomers showed marked increases in measures of cytotoxicity i.e. increase in cellular death (as documented in the cell culture section) and increases in LDH production and release observed in cultured macrophages. This apparent contradiction to *in vivo* studies is likely to be due to overestimation of the toxicity in relatively stagnant culture conditions. The overall results of the cellular and biochemical tests indicated that PHB, P(HB-HV) and their degradation products were well tolerated in soft tissue and therefore biocompatible with this tissue.

Protein adsorption to polymer surfaces was assessed as an indication of the potential of a material to be thrombogenic (chapter 5). Due to limited clinical experience *ex vivo* and *in vivo* work could not be done. The data collected in this work showed that PHB preferentially adsorbed albumin to its surface and that platelet adhesion to coated and uncoated polymer was minimal. Such findings with reference to well documented blood compatible polymers, indicate that PHB may be a nonthrombogenic material. P(HB-HV), preferentially adsorbed IgG and adsorbed a greater number of platelets indicating its potential thrombogenicity.

Each aspect of the inflammatory response used to assess biocompatibility has recognised limitations as described in the individual chapters. However by using a range of experiments a substantial picture has been formed on the effects on the physiological environment of the implantation of PHB, P(HB-HV) and their degradation products. Aspects of the inflammatory response and the adsorbed plasma protein profile correlated well and a comprehensive picture of the biochemical events and cellular changes

following implantation/injection was obtained.

The biocompatibility of these biodegradable polymers has been assessed exclusively in the soft tissue assessing the magnitude and duration of the inflammatory response. A number of parameters were standardised throughout the toxicity evaluation programme including the animal model used and the physical form of polymer presentation in culture or *in vivo*. The rat was used throughout all *in vivo* tests and was the animal of choice, as inbred strains of the male Wistar rat can lead to good reproducibility over many generations and allowed comparisons to be made. The number of animals required per experiment was large and the use of rabbits as the animal model would have been expensive. The main disadvantage of the rat was its relatively short life span, 18-24 months, which did not allow long term measurements of biocompatibility, such as the assessment of carcinogenic effects. However use of two animal models would have been useful as species respond differently to the same stimuli (Williams, 1986). In general the mononuclear cell predominates in the rabbit whereas the PMN appears with greater frequency in the rat. It is usually assumed that man is less sensitive than the rabbit or may be better equipped to cope with the introduction of a foreign material than some of the lower species. The physical form of the polymers was standardised, as either smooth films or microspheres of approximate diameter 40µm. It is well known that different physical forms of the same material may differentially influence the inflammatory reaction to these materials (Wood, 1970; Sevastjanova *et al.*, 1987; Bakker *et al.*, 1988). The implantation site was standardised in soft tissue, but implantation site is known to affect the degree of inflammation seen (Hench and Etheridge, 1982; van Blitterswijk *et al.*, 1986).

Further studies using variations in implantation site, animal

model, physical forms and size of the implant, and in the case of the copolymer different mole fractions of HV, are needed in order that the assessment of biocompatibility of PHB and P(HB-HV) can be conclusive. However preliminary studies *in vitro* and *in vivo* on the biocompatibility of these polymers and their use as biodegradable drug delivery systems suggest their potential.

Appendix 1

## Microsphere production.

Microspheres were produced by a solvent evaporation process at 22°C and at atmospheric pressure. The standard procedure involved dissolving the required amount of polymer in chloroform by reflux. The mixture was poured rapidly into an aqueous phase containing 2% v/v polyvinylalcohol (PVA) as an emulsifier and stirred at a constant rate of 1400rpm. The resulting emulsion was agitated at 22°C for 24 hours during which the chloroform had evaporated. The partially dried microspheres were allowed to settle and the aqueous phase containing the polymeric dispersing phase was replaced with distilled water. When all the chloroform had evaporated the microspheres were collected and isolated by centrifugation. They were then washed and dried in vacuo at 45°C for 24 hours then stored ready for use.

## Film production.

4% w/v polymer solutions were prepared by refluxing in redistilled chloroform. Polymer solutions were cast onto clean, dry glass plates using a thin layer chromatography (TLC) applicator. The clearance setting of the TLC applicator was chosen to produce dry films of thickness 90-100µm. The resultant cast films were dried slowly in draught free conditions for 2 hours and then overnight in a vacuum oven at 45°C. Films were stored at room temperature one week prior to use.

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